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EXTRAPOLATION OF INHALED PARTICULATE TOXICITY DATA FROM EXPERIMENTAL ANIMALS TO HUMANS

FINAL REPORT

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EXECUTIVE SUMMARY

Significant progress has been made over the past three years to develop methodology and assess various tissues and tissue sensitivity endpoints with the ultimate goal of validating a proposed extrapolation model. This model will allow the quantitative extrapolation of inhaled particulate toxicology data from experimental animals to man. Methodology was developed to accurately measure nucleotide levels in small tissue samples, to determine cellular toxicant levels, to isolate tissue or cells at various levels of the respiratory tract, and to culture animal and human cells for identical treatment conditions. The tissues assessed were nasal turbinate epithelial, olfactory epithelial, and alveolar macrophages. Nasal and pulmonary lavage fluids were also studied. The tissue sensitivity endpoints studied were cellular energy charge, ascorbic acid, and glutathione levels and single-stranded DNA breaks. In these studies we found that human nasal turbinate epithelial cells contained eight to ten times more cadmium than rat cells following a 2-h *in vitro* exposure to 1 mg/mL cadmium. Although the human cells contained more cadmium per cell, the energy charge was not significantly lower than rat cells until after 4 h of exposure. *In vivo* exposures induced slight reproducible decreases in energy charge (1-5%), and the cells contained small amounts of cadmium. Because the *in vivo-in vitro* differences in cellular dose and energy charge were great, we could not accurately estimate the extrapolation ratio. Using DNA damage as an endpoint, the extrapolation ratio for *in vitro* exposure between human and rat macrophages was 1.28; therefore, an *in vitro* animal to *in vitro* human correlation became feasible with rat and human alveolar macrophage cells for DNA damage. This part of the model could then provide quantitative extrapolation values for other compounds of interest. These comprehensive, comparative animal to human extrapolation studies were unique in that, for the first time, tissue response was measured as a function of actual cellular dose, and common endpoints were used for the same target cell types in different species. These studies provided most of the necessary groundwork leading up to the validation of the model. The *in vitro* to *in vivo* animal correlation will require a small amount of additional effort to establish cellular dose ranges that overlap. These studies have also provided data that strongly suggest that animal and human cells can differ significantly in both uptake and response to a given toxicant. Because of these data, it is recommended that more effort be expended to understand these differences and quantitate them. The described cell sensitivity model could then be used to provide quantitative animal to human extrapolation values for inhaled particulates, which will be extremely valuable for human risk assessment.

FOREWORD

Technical support services for this study were provided by Northrop Services, Inc., under U.S. Environmental Protection Agency (USEPA) Contract No. 68-02-4032 from July 1984 to October 1987. This work was conducted in response to Technical Directives 4.5.6, 4.5.7, 4.5.8, and 4.4.3 (7/84 to 9/87). Chemical analysis support services were provided by Industrial and Environmental Analysts, Inc.

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. [NIH] 85-23, Revised 1985).

For the protection of human subjects, the investigator(s) have adhered to policies of applicable Federal Law 45CFR46.

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INTRODUCTION

The U.S. Army conducts an animal toxicology program to evaluate potential health risks to troops, thereby permitting development of control strategies to reduce those risks. If animal toxicological results could be quantitatively extrapolated to humans, the control strategies could be more precise, facilitating training regimens optimized for the lowest health risks. Such an extrapolation is, at present, technically feasible for pulmonary effects of inhaled particles which do not undergo biotransformation to active metabolites. The research reported herein was designed to provide a model to test this technique, which when combined with existing research programs in dosimetry, will permit a quantitative extrapolation. The validation of this research approach also provides the basic foundation to permit the design of future investigations to address a large series of effects and classes of chemicals.

Extrapolation requires species comparisons of both dosimetry and tissue sensitivity between species. This project is mainly concerned with the latter. Tissue sensitivity is defined as the degree of toxic response observed in the organ or cells. The present study examines the relative sensitivity of respiratory tract tissues in different animal species and attempts to address the question of tissue sensitivity in humans by use of the parallelogram principle shown in Figure 1.

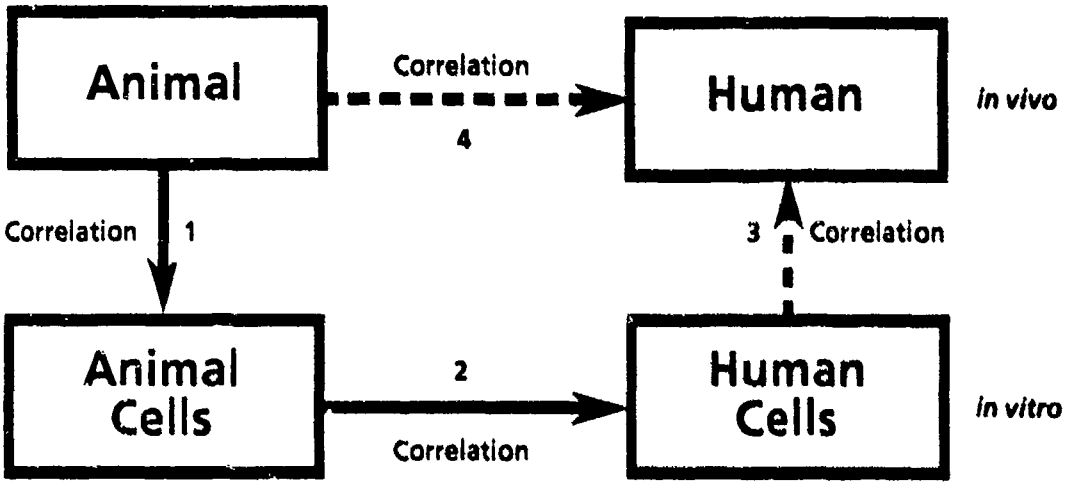

Figure 1. The Parallelogram Model for Extrapolating Animal Toxicology Data to Humans. ———, experimental correlations; – – – –, theoretical correlations.

The desired extrapolation (correlation 4) is between *in vivo* animals and *in vivo* humans. Since it is not ethically possible to perform controlled human exposures using the toxic particles, it is necessary to bridge this gap by establishing the validity of an *in vitro* to *in vivo* extrapolation

(correlation 1 and presumably correlation 3) through the use of laboratory animals, and then by making comparisons of animal and human cells using tissue culture (correlation 2). The basis for making correlations between 1 and 2 will be the comparison of dose-effect curves where both dose and toxic effect is quantified at the tissue level. This can be done if easily detected particles are used and toxicity endpoints applicable to tissue collected both *in vivo* and *in vitro* are selected. If *in vivo* and *in vitro* species sensitivities to a particle are equal, the dose-response curves at the tissue level should be superimposable.

Nasal epithelial tissue was chosen for initial studies because (1) the respiratory epithelium of the nasal cavity is similar to the epithelium lining the entire respiratory tract, for which other toxicity data exist; (2) nasal tissue in animals and humans is typically exposed to a variety of aerosols; (3) cell culture methods for nasal epithelium were available; and (4) normal human nasal epithelial tissue were available from reconstructive surgeries.

A heavy metal with a high degree of toxicity was chosen initially for use in this project, as it is easily detected in tissue at low concentrations and has relevance to environmental exposures. Consultation with engineers about generating aerosols of heavy metals led to the conclusion that cadmium sulfate ($CdSO_4$) would be a good starting compound.

Cadmium sulfate is not metabolized (thereby avoiding species differences in metabolism) and is highly soluble in culture medium. Also, *in vitro* exposure to Cd has been reported to significantly affect adenine nucleotide levels in alveolar macrophages (AMs) (Waters, 1975) and in isolated hepatocytes (Muller and Ohnesorge, 1984). Our preliminary experiments demonstrated that $CdSO_4$ was more toxic than $ZnSO_4$, $NiSO_4$, or CrO_3 (Figure 2). In other studies (Tan, 1984), $CdCl_2$ was found to be more toxic than 15 other metallic compounds for Chinese hamster ovary cells exposed *in vitro*.

Cadmium "dose" was measured by determining the intracellular levels following *in vitro* or *in vivo* exposure. The Cd content of cultured nasal turbinate epithelial (NTE) cells and of NTE tissue from exposed animals was determined and correlated with the concentration of Cd added to the cell cultures, or with the parts per million concentration of Cd that the animals inhaled.

In order to compare the toxic responses following particulate exposure of tissues from whole animals and cultured cells, an endpoint was needed that could be applied to both models. Adenine nucleotide levels and energy charge (EC) were measured as cytotoxicity endpoints because they appeared to be good quantitative indicators of early cell injury (Muller and Ohnesorge, 1982) and could be easily correlated from *in vitro* to *in vivo*.

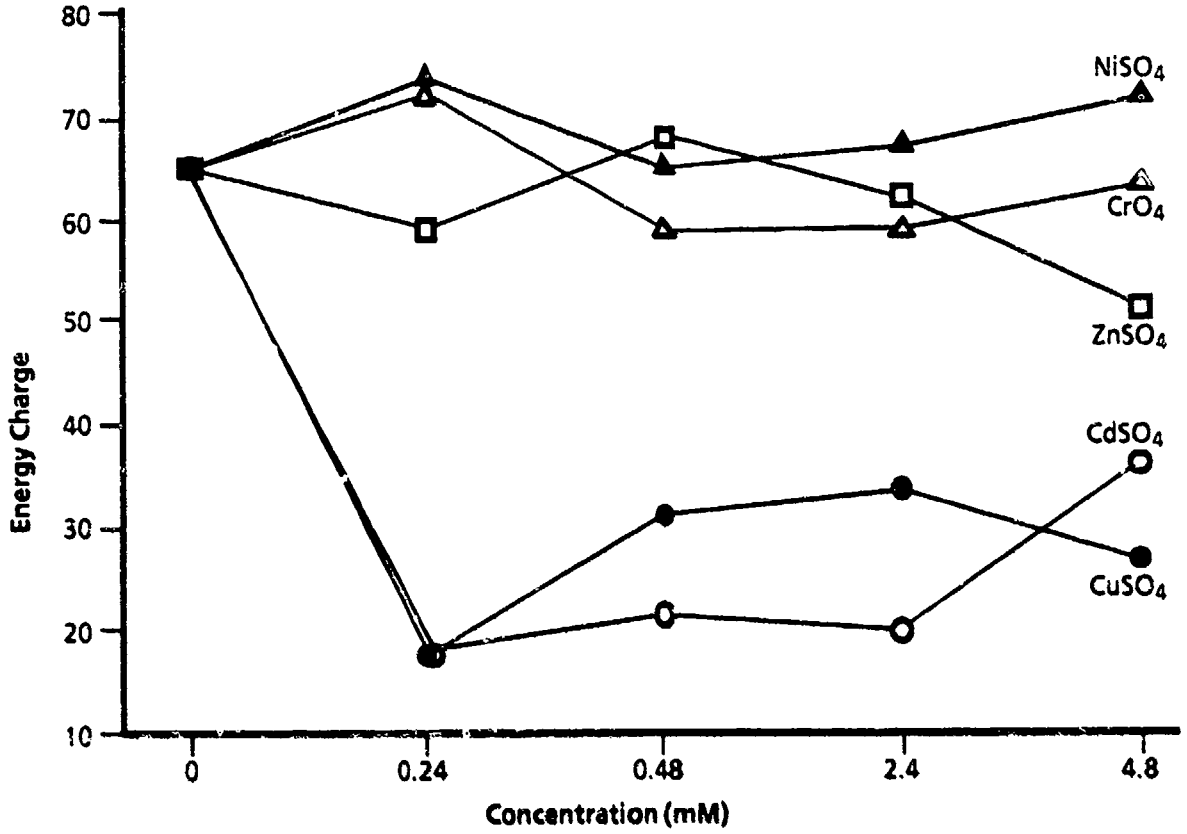

Figure 2. Effects of Metals on Energy Charge of Rat Nasal Turbinate Epithelial Cells. The cells were extracted 4 h after the end of a 2-h exposure.

Considerable effort was directed toward evaluating and developing methodology for accurately determining adenine nucleotide pools in extracts from tissues and cultured cells in an attempt to use these data for indexing Cd toxicity. Previous studies at the EPA demonstrated that Cd exposure reduces the adenosine triphosphate (ATP) levels in AMs with an ED_{50} of 7.4×10^{-5}M Cd (Waters, 1975). Other studies showed ATP reduction in lungs of rats exposed for 4 h to the pulmonary irritant, phosgene (Currie et al., 1987).

Adenosine triphosphate must be expressed in terms of some denominator that is equally applicable to both *in vivo* tissues and *in vivo* cultures. Wet or dry tissue weight, protein, and DNA were considered as denominators, but each had limitations. Dry tissue weight was difficult to determine in cultured cell preparations. Protein concentration, although proportional to cell mass, is not proportional to cell number. In addition, protein is present in mucus and is used to coat tissue culture flasks to enhance cell growth *in vitro*. Protein concentration is also altered by edema and would therefore be difficult to accurately measure at the sensitivity needed. In contrast, DNA content was useful since it is proportional to cell numbers and can be obtained for cells exposed *in vitro* and for tissues from exposed animals. For this reason, nucleotide data were expressed using

DNA as the common denominator for *in vitro* and *in vivo* data. Both rat and human NTE cells contain similar amounts of DNA/cell (Table 1).

TABLE 1. DNA AND TOTAL SULFHYDRYLS IN RAT AND HUMAN NASAL EPITHELIAL CELLS

Nasal Epithelium	DNA (pg/cell)[a]	Sulfhydryls (nM/mg protein)[a]
Human	6.0	1,163 ± 166[b]
Rat	6.2	799 ± 220

[a] Means values ± SD, n = 3 cultures.
[b] Significantly greater than corresponding values for rat cells ($p<0.05$).

In addition to using DNA as a denominator, total nucleotide concentrations were used in calculations of EC. Energy charge is a unit-less number ranging from 0 to 1 that reflects the level of energy available to the biological system. Atkinson (1968) proposed a theory based on the following two primary enzymatic reactions involved in ATP synthesis:

1. The reaction catalyzed by adenylate kinase

$$ATP + AMP \leftrightarrows 2ADP$$

where AMP is adenosine monophosphate and ADP is adenosine diphosphate

2. Adenosine triphosphate regeneration by electron transport phosphorylation, or otherwise, or ATP utilization in metabolism.

$$2ADP + 2Pi \leftrightarrows 2ATP + 2H_2O$$

Combining these two reactions, they proposed an overall energy relationship that was:

$$EC = \frac{[ATP] + \quad 0.5\,[ADP]}{[ATP] + \quad [ADP] + [AMP]}$$

When in a fully charged state, ATP levels represent 100% of the adenine nucleotide pool and the EC value approaches 1.0. Normal cell populations on tissues are thought to maintain an EC level of approximately 0.80.

To investigate an additional endpoint for the extrapolation of inhaled toxicants, an indicator of DNA damage was chosen. The alkaline elution method was adapted to evaluate DNA damage induced *in vitro* and *in vivo* by $CdSO_4$. The alkaline elution technique utilizes differential filtration rates of DNA to discriminate DNA single-strand sizes. The filters do not adsorb DNA under the conditions employed, but rather, act mechanically to impede the passage of long DNA strands. Variations of the technique can be used to measure single-strand breaks, alkali-labile sites, DNA-protein crosslinks, and DNA-interstrand crosslinks. The hypothesis behind this technique is that genotoxic agents cause DNA strand breaks, and under alkaline conditions, the DNA denatures from double strand to single strand. Strands with breaks pass through the filter more quickly because

they are smaller, whereas unbroken strands are longer and, hence, take longer to pass through the filter. The sensitivity of the measurement is on the order of one DNA lesion per 10^7 nucleotides.

The goals and objectives of this project were to (1) determine the relationship of *in vivo* animal (exposed by inhalation) and *in vitro* animal responses; (2) determine and relate *in vivo* and *in vitro* cellular responses to a cytotoxic particle in several animal species; (3) determine the relationship of *in vitro* animal to *in vitro* human responses; (4) compare and relate particle dose-response data using common cytotoxicity endpoints in cultured animals cells versus cultured human cells; (5) extrapolate from *in vitro* human response to *in vivo* human response; (6) extrapolate the potential human response from the *in vivo* animal inhalation data; and (7) apply regional pulmonary deposition and clearance data comparisons between animals and humans prior to making species sensitivity judgments as part of the extrapolation of *in vivo* animal results to man.

METHODS

A considerable amount of effort was devoted to methods development for this project because very few sensitive techniques were available to directly measure and correlate the absorbed tissue dose of a toxicant with a common toxicity endpoint in the same tissue sample. In addition, methods were required that were directly applicable to both *in vivo* and *in vitro* models and for different species.

NASAL EPITHELIAL CELL ISOLATION PROCEDURES

Human Nasal Epithelial Cells

Human nasal tissues were provided by the University of North Carolina (UNC) School of Medicine. Tissues were from healthy patients undergoing cosmetic surgery, and unless notified otherwise, only lateral nasal turbinate tissue was provided.

The immediate isolation of NTE cells by enzymatic dissociation of tissue resulted in only $1 - 5 \times 10^5$ cells per tissue sample. However, a greater number of cells could be obtained by cutting tissues into 2-mm^2 explants and placing them onto the surface of tissue culture dishes to produce outgrowths. The NTE cells grew out from the explants onto the culture dish. These primary cultures were then dissociated with trypsin-EDTA and replated. Four to five outgrowths per explant (10-15 explants per tissue sample) provided about 10^6 cells. This methodology proved successful in providing adequate cell numbers for several experiments with limited tissue availability.

Rat Nasal Epithelial Cells

Rat NTE cells can be isolated by three different techniques: (1) *in situ* enzymatic dissociation, (2) dissociation of surgically dissected tissues, or (3) by an explant/outgrowth method. In initial studies, rat NTE cells were isolated by *in situ* enzymatic dissociation; however, because human NTE cells were only obtained by the explant/outgrowth method, this method was also utilized for rat NTE cells. Male, Fischer 344 rats (F-344), six to eight weeks old were sacrificed by CO_2 euthanasia and the respiratory turbinates were removed surgically. The pieces of tissue were cultured and the NTE cell outgrowths trypsinized and replated by the same method used for human tissue.

The cells isolated from human and rat tissue were characterized as epithelial on the basis of morphology using light and electron microscopy and due to the presence of large amounts of keratin protein, a major structural protein found in significant quantities only in epithelial cells.

NASAL EPITHELIAL CELL CULTURE METHODS

Rat NTE cells grew best when plated in Ham's F12 medium containing five growth factors and 1% bovine serum albumin (BSA). Human NTE cells were grown in Ham's F12 medium containing six growth factors without BSA. By modifying the amounts of the six growth factors and supplementing with 12.5% 3T3-conditioned medium, a culture medium was developed that provided for good growth of both human and rat NTE cells. These differences in growth medium were eliminated to avoid the possible contribution to differences in cell sensitivity to toxicants.

Rat and human NTE cells were plated on 60-mm tissue culture dishes in 3 mL of growth medium. Because the plating efficiency of rat NTE cells was two- to threefold lower than that of human NTE cells, a higher plating density (two- to threefold) was used for rat NTE cells. In addition, 1% fetal bovine serum (FBS) was added to the rat cell plating medium for 24 h to facilitate cell attachment. Using these methods, a sufficient number of rat NTE cells attached for use in Cd experiments.

ALVEOLAR MACROPHAGES

Rat AMs were obtained by a lung lavage procedure. Male F-344 rats were sacrificed by halothane inhalation followed by exsanguination. The trachea was immediately cannulated and the lungs filled with 37°C saline (35 mL/kg body weight). The lavage fluid was withdrawn from the lung and placed in a tube on ice. The lavage procedure was repeated three times, using fresh saline each time.

The four lavage fluid samples containing AMs were pooled for each rat. After centrifugation, the supernatant (lavage fluid) was removed for Cd and protein analysis. If the AMs were from *in vivo* exposed rats, the pellets were resuspended in 0.5 to 1.0 mL cold 3% perchloric acid ($HClO_4$). The $HClO_4$ samples were then prepared for analysis of nucleotides, antioxidants, DNA, and Cd by the same procedures used for NTE cells. Alveolar macrophages to be used for *in vitro* Cd exposures were resuspended in Dulbecco's modified Eagle medium (DMEM) containing 5% BSA. After obtaining a cell count, the AMs were dispensed into 15-mL tubes and the desired amount of $CdSO_4$ was added. After exposure, the AMs were analyzed for nucleotides, Cd, antioxidants, and DNA by the same methods used for NTE cells.

Human AMs were obtained from an ongoing EPA project at UNC - Chapel Hill. Human lavage fluid was collected from normal subjects using a fiberoptic bronchoscope which was inserted under local anesthesia. Six 50-mL aliquots of saline were sequentially injected and withdrawn from the right middle lung lobe, then from the lingula of the left lung. The lavage fluid was immediately placed on ice. Human AMs were prepared for Cd exposures using the same methods utilized for rat AMs.

CELL VIABILITY

Cell viability was determined by the trypan blue dye exclusion assay. A 20-mL aliquot of cell suspension was mixed with an equal volume of 0.1% trypan blue. Nonviable cells were stained while viable cells excluded the dye. Total and nonviable cell counts were performed using a hemocytometer.

ANTIOXIDANT ASSAY

Glutathione (GSH), ascorbic acid, and uric acid levels were measured in $HClO_4$ extracts of cells and tissues by a reverse-phase high performance liquid chromatography (HPLC) method. The $HClO_4$ extracts were injected directly into the HPLC and eluted from a C18 column with an isocratic mobile phase containing 0.025 M Na acetate and 0.05 M Na phosphate buffer, pH 4.8, 0.005 M octylamine, and 18% methanol. The electrochemical detector (ESA Inc., Bedford, MA) was set to 0.6 volts relative to Ag/AgCl potential. A sample chromatogram of nasal epithelial tissue is shown in Figure 3. Ascorbic acid and GSH were identified by the similarity of their retention times with those of standards. Procedures used were those of Allison and Shoup (1983) and Kutnink *et al.* (1985).

SULFHYDRYL ASSAY

Total cellular sulfhydryl levels were measured in rat and human NTE cell cultures by the method of Sedlak and Lindsay (1968).

ALKALINE ELUTION

The alkaline elution technique utilized 0.2 µm pore size polycarbonate filters (Nucleopore) to discriminate DNA strand sizes in cells. The filters do not absorb DNA under the conditions employed, but rather, act mechanically to impede the passage of long-stranded DNA.

The isolated cells were resuspended in 2 mL of phosphate buffered saline (PBS) and held on ice until the elution column was prepared. To prepare the column, the polycarbonate filter was wetted with 0.01 M Na_2EDTA, pH 9.6, placed in the Millipore holder, and rinsed with 10 mL of ice cold PBS. The cells were then added to the column, and the PBS was allowed to pass through the column until about 1 mL remained. Then 5 mL of lysis buffer (2% sodium lauryl sulfate, 0.05 M Tris base, 0.05 M glycine, 0.025 M Na_2EDTA, pH 9.6) was added. The lysis of cells proceeded for 48 h in the dark. At the end of this period, approximately 10 mL of 0.06% lauryl sarcosine in 20 mM Na_2 EDTA was passed through the column. The DNA remaining on the filter was then eluted with approximately 2% tetrapropyl ammonium hydroxide in 20 mM EDTA, pH 12.1. The elution rate was 0.035 mL/min, and seven to ten 90-min fractions were collected. The fractions (approximately 3 mL) were mixed with 3 mL of a 60 µg/mL Hoechst 33258 solution. The sample readings were compared to a calf thymus DNA standard curve and the amount of DNA per sample calculated. The percent DNA remaining on the filter was calculated and differences between treatment groups determined.

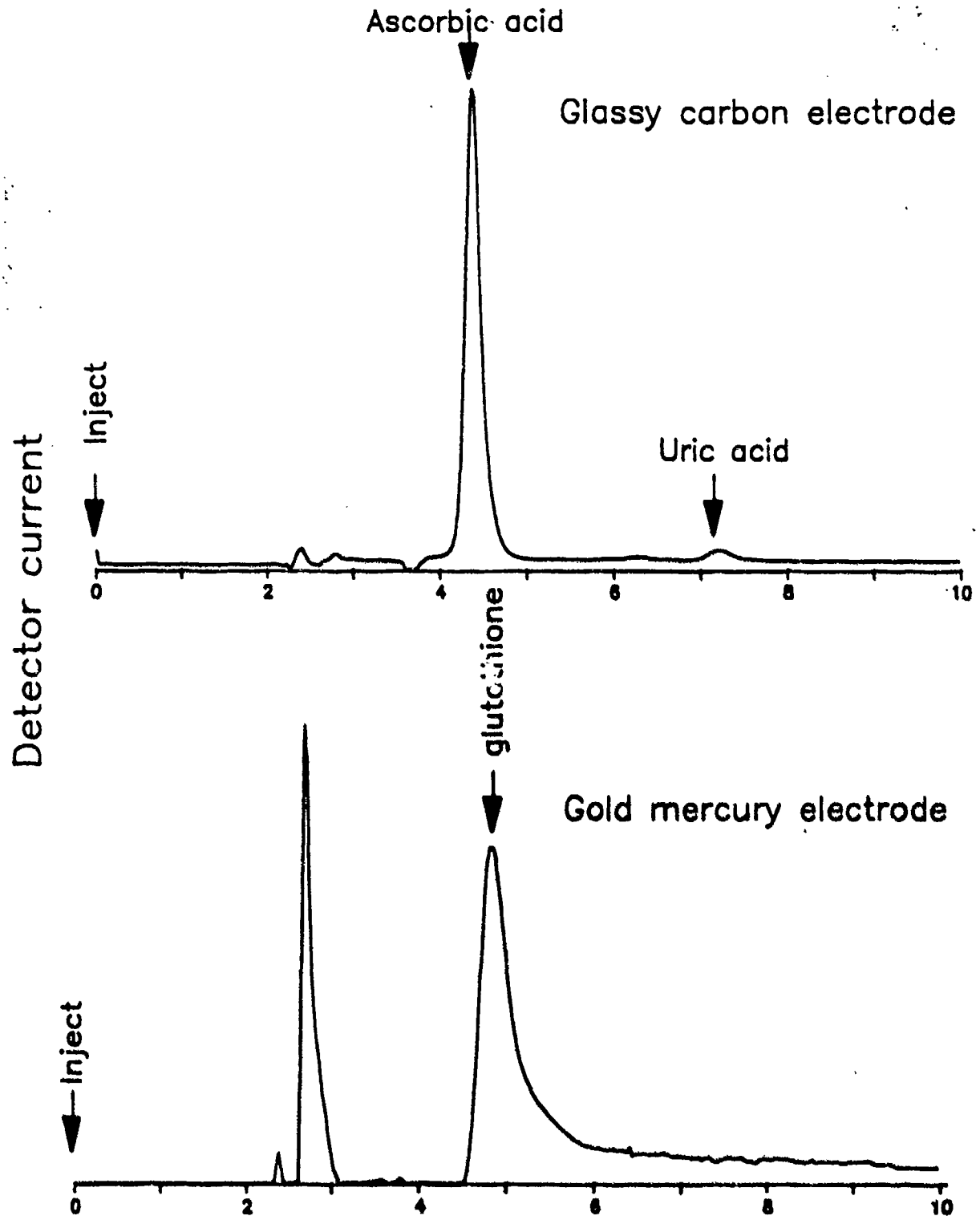

Figure 3. Typical High Performance Liquid Chromatog ns of Ascorbic Acid and Glutathione.

CADMIUM ANALYSIS

The most sensitive method for analysis of Cd was determined to be graphite furnace atomic absorption (GFAA) spectrophotometry. The GFAA analyses of Cd were performed by a contract laboratory. In an initial study, background levels of Cd in reagents, culture media, and glassware were found to be insignificant.

To test the feasibility of the method for Cd sample preparation, and to provide test samples containing a cell matrix for GFAA analysis, 5×10^4 NTE cells spiked with known amounts of Cd were prepared and analyzed for total Cd. The protocol for sample preparation was found to be feasible to analyze cellular Cd content. The GFAA method for analyzing cellular Cd proved to be highly sensitive and reproducible for low levels of Cd up to about 10 ng, with some loss of linearity at higher levels (> 100 ng). A standard curve, spiked samples, and 3 to 4 duplicates were run for each group of 15 to 25 samples analyzed as a quality control check.

EXPOSURE METHODS

Every effort was made to utilize the same methods for the *in vitro* and *in vivo* techniques throughout this project; however, it was not technically feasible to use the same exposure method *in vitro* and *in vivo*. Cultured cells were exposed to $CdSO_4$ in aqueous solution, while rats were exposed to a $CdSO_4$ aerosol.

In Vitro Cadmium Exposures

Nasal Epithelial Cells

Nasal epithelial cell cultures were rinsed with PBS to remove unattached cells and growth medium before adding the $CdSO_4$ exposure medium. Cultures were exposed in an incubator at 37°C, 5% CO_2, and 95% humidity. Immediately after exposure, the medium containing $CdSO_4$ was removed and the cells were rinsed three times with 37°C PBS to remove the excess $CdSO_4$. Because *in vivo* exposures were limited to 2 h, this duration was also used for *in vitro* exposures.

Alveolar Macrophages

For *in vitro* studies, AMs were exposed to $CdSO_4$ while in suspension, rather than while attached to tissue culture dishes. Immediately after isolation by lavage, AMs were resuspended in medium (DMEM) containing 5% BSA. This medium was used to maintain AM viability and to help prevent cell attachment (Ca^{2+},Mg^{2+}-free). After a cell viability count was performed, AMs were transferred to 15 mL centrifuge tubes, the desired amount of $CdSO_4$ was added, and cells were incubated for 2 h. Each tube was gently vortexed every 15 min to prevent AM attachment during the 2-h exposure. After exposure, each tube was gently vortexed and a 40-µL aliquot taken for viability

counts. The remainder of the AM suspensions were prepared for nucleotide, Cd, and alkaline elution assays.

In Vivo Cd Exposures

To restrict Cd primarily to the respiratory tract during *in vivo* exposure, a nose-only exposure system was selected. The chamber consisted of a modular polyvinyl chloride column that could be altered to hold the desired number of animals. Animals were placed in cylindrical plexiglass holders, and either placed on the laboratory bench (controls) or inserted into outlet ports on the chamber modules. Aerosols were generated from an aqueous $CdSO_4$ solution using a nebulizer. Chamber $CdSO_4$ concentration was controlled by nebulizing solutions of different $CdSO_4$ concentrations.

Advantages to these new chambers were that they were easy to clean; they had a separate aerosol supply for each animal so that no re-breathing occurred; their columnar shape made particle distribution more controllable; and the small modules resulted in use of small volumes of air and, hence, less expense for chemicals.

During testing of the chamber, filter samples were taken to determine the chamber concentration of Cd in milligram per cubic meter. Cascade impactor samples were obtained to ascertain the particle size and size distribution. These test samples were solubilized in nitric acid and analyzed by atomic absorption. The mass median aerodynamic diameter (MMAD) and geometric standard deviation (sg) were calculated from cascade impactor samples. The MMAD of particles produced by the aerosol generator ranged from 0.5 to 0.6 mμ with an sg of around 1.5.

To evaluate the distribution of $CdSO_4$ within the chamber, filter samples were obtained from different rat ports and analyzed for Cd. The aerosol distribution was found to be uniform for all sections of ports in the chamber, except for the top two sections. The lower concentration of Cd in these top sections was due to inadequate mixing of the aerosol and diluent air entering the top of the chamber. This mixing problem was corrected by allowing the aerosol and diluent air to mix more thoroughly before entering the chamber.

Nose-only exposures were limited to 2 h because of heat build-up in the holding tubes and possible stress to the animals. Because the exposure duration was limited to a maximum of 2 h, and the particle size restricted to 0.5 to 0.6 μm for the available aerosol generator, the Cd dose could only be increased by increasing the Cd concentration (mg/m^3).

In initial range-finding studies, rats were exposed to 0.44 mg CdO/m^3 for 2 h and the Cd levels were measured in the NTE tissues. Cadmium deposition in NTE tissues from this dose was below detection limits. In subsequent exposures, it was determined that Cd concentrations greater than 1.0 mg/m^3 were required to produce detectable deposition in the nasal tissues.

SAMPLE PREPARATION AND NUCLEOTIDE EXTRACTION

Methods were developed for the extraction and analysis of the adenine nucleotide pool from small tissue samples and cultured cells. Rat nasal epithelial tissue samples ranged in weight from 20 to 40 mg wet weight per animal. Cultured cells numbered from 0.6 to 2.0×10^6 cells per 6-cm culture dish.

Because the enzymatic breakdown of ATP occurs rapidly after cell death or injury, it was essential to inactivate these degradative enzymes immediately after $CdSO_4$ exposure. Cold perchloric acid ($HClO_4$) was used as the "stopping reagent" during *in vitro* assays. High concentrations of $HClO_4$ have been shown to cause the breakdown of nucleotides; however, a 3% aqueous solution of $HClO_4$ was shown to be effective in nucleotide assays. Perchloric acid extracts were kept cold at all times to help prevent residual enzymatic and nonenzymatic breakdown of nucleotides.

After *in vivo* exposures, it was necessary to euthanize the animal and dissect out the nasal epithelial tissue before extracting with $HClO_4$. Nucleotides were found to degrade rapidly during the time between euthanizing and $HClO_4$ extraction. A method of euthanizing animals was required that would immediately inactivate degradative enzymes. Two methods were evaluated: (1) decapitation into liquid nitrogen and (2) focused microwave irradiation. Euthanizing by microwaves was much more effective in preserving ATP levels in NTE tissues. The ATP to AMP ratios and EC values were significantly higher for rats euthanized by microwaves than for rats euthanized by decapitation (Table 2). Immediately after exposure, rats were euthanized by focused microwave irradiation (2.2 s, high power), and then the NTE tissues were surgically removed and placed on ice. Tissue samples were then weighed and homogenized in 1.0 mL cold 3% $HClO_4$.

TABLE 2. EFFECTS OF MICROWAVING VS. DECAPITATION ON NUCLEOTIDE LEVELS IN RAT NASAL EPITHELIUM[a]

Euthanasia Procedure	ATP:AMP	Energy Charge
Microwaved[b]	3.57[c]	0.68[c]
Decapitated	0.66	0.42

[a] Air-exposed control rats
[b] Focused microwave irradiation, 2.2 s, high power
[c] Significantly different at $p < .05$.

Perchloric acid extraction (0 to 4°C) of the samples was followed by centrifugation to remove precipitated protein and DNA. The resulting supernatant, containing the full complement of nucleotides, was stored at -80°C until analyzed. At that time, the solution was neutralized with $KHCO_3$, the resulting precipitate removed by centrifugation, and the sample directly analyzed by HPLC.

HPLC ANALYSIS OF NUCLEOTIDES

Cell or tissue $HClO_4$ extracts were quickly thawed in a 37°C water bath and 50 to 110 µL aliquots transferred to sample vials containing 10 µL of xanthosine (10 ng/µL). Xanthosine was used as an internal standard because it is not normally present in nasal epithelial cells and is easily resolved from the nucleotides being measured. Samples were thawed and analyzed in groups of three because some decomposition of ATP was observed after approximately 2 h at room temperature. Samples (50 to 100 µL) were injected into the HPLC by a Waters WISP autosampler.

The mobile phase consisted of a linear gradient from 100% buffer A (containing 0.15 M $NH_4H_2PO_4$, 1.2% CH_3CN, pH 6.0) to 100% buffer B (containing 0.09 M $NH_4H_2PO_4$, 9.4% CH_3CN and 9.4% CH_3OH, pH 6.0) over a 23-min period. The flow rate was 0.8 mL/min. Buffers were prepared fresh each day from deionized, distilled water and HPLC-grade solvents. Buffers were filtered (0.45 um filter) and degassed with helium before use. To prevent evaporation of the small amount of CH_3CN, buffer A was filtered and degassed before addition of CH_3CN, and both buffer reservoirs were sealed after degassing.

The separation of nucleotides was achieved using a spherisorb C18 column, 250 x 4.6 mm, 5 µm particle size (Regis Chemical Co.). The column was re-equilibrated to buffer A for 15 min after each sample run. Nucleotides were detected by UV absorption at 254 nm. Nucleotide peaks were integrated by a Sigma 15 integrator and peak areas entered into Lotus 1-2-3 spreadsheets for calculations. These procedures are a modification of Wynants and Van Belle (1985).

DNA DETERMINATIONS

Pellets from the perchloric acid extraction of NTE cells and tissues contain proteins in addition to cellular DNA. In our attempts to correlate data between tissue samples and *in vitro* cell cultures, we were interested in an accurate micro-technique for DNA determination. Such a method has been described which utilizes the fluorochrome Hoechst 33258. Extracted pellets were resolubilized in pH 12 solutions of NH_4OH and then neutralized with HCl to a final pH of between 6 and 7. Solubilized samples were then diluted with one volume of 2 M NaCl. At this ionic strength, DNA-protein association was minimal. The Hoechst dye was then added in $NaPO_4$ buffer (0.05 M, pH 7.4) to a final concentration of 1 µg/mL. Dye reaction with the DNA was maximal after 2.5 h at 25°C. The dye-DNA complex, when excited at 356 nm, has an emission maximum at 458 nm. A linear response of fluorescence intensity to calf thymus DNA was demonstrated in the range of 0.6 to 15 µg of DNA/mL with a minimum detection limit at 10 ng/mL, and served as the standard curve that was performed in each assay. These procedures were modified from Downs and Wilfinger (1983).

NASAL LAVAGE

Because nasal lavage could provide a rapid and easy method of detecting injury (possibly tissue dose) to the nasal epithelium, some effort was directed toward exploring this methodology. It is very difficult to remove only the very thin epithelial layer of nasal cells; therefore, several new nasal lavage techniques that extract the nasal epithelial nucleotides *in situ* were tested. In a preliminary experiment, rats were anesthetized with ketamine + xylazine and the trachea exposed. The trachea was then severed and two cannulas implanted; one to the lungs and one to the nasopharynx. The cannula to the lungs allowed the animal to breathe, while solutions were perfused into the nose through the other cannula. The nasal passages were first rinsed with PBS, which removes about 95% of the mucus covering the epithelium (Dr. Kevin Morgan, Chemical Industry Institute of Toxicology, personal communication). The nasal epithelial cell nucleotides were then extracted *in situ* by perfusing the nasal passages with cold 3% $HClO_4$.

To eliminate problems with loss of lavage fluid into the esophagus and blood contamination, a new technique was developed that entailed constructing a combined tracheal cannula and a nasal lavage tube. A plastic cast of the rat naso-pharyngeal cavity was used as a model to make this tube. When inserted via the mouth into the trachea of the anesthetized rat, the tube allowed the animal to breathe through its mouth while the nasal cavity was lavaged with phosphate buffer or cold 3% $HClO_4$. The animal remained alive during this procedure, thus avoiding degradation of nucleotides. The $HClO_4$ extract was collected via the nostrils of the rat and analyzed directly for nucleotides and antioxidants.

This new technique was used to determine if the effects of $CdSO_4$ could be detected when extracting nucleotides *in situ*. Two groups of rats were exposed to 10 mg/m^3 for 2 h and nasal lavage performed at 0 and 24 h after exposure. Nasal lavage fluid (PBS and $HClO_4$) were analyzed for nucleotides, Cd, and ascorbic acid.

STATISTICS

Statistically significant differences between groups ($p < 0.05$) were determined by Student's t-tests using pooled variance from one-way analysis of variance. Where variance differed more than tenfold between groups, analyses were performed on log-transformed data.

IN VITRO-EXPOSED NASAL EPITHELIAL CELLS

Exposure Methods

Unlike human NTE cells, rat NTE cells must be plated on collagen-coated dishes for optimal attachment and growth. However, the requirement of a collagen substratum by NTE cells was found to interfere with the nucleotide analyses. The collagen substratum that was removed with the NTE cells during the scraping process was not removed by the extraction method, and subsequently, blocked the flow of the HPLC column. Efforts to replace the collagen with another substratum or to remove the collagen from cell samples by acid precipitation and filtration were unsuccessful. Enzymatic removal of cells from the collagen-coated dishes appeared to eliminate the collagen contaminants; however, the use of enzymes adversely affected the cellular adenine nucleotide levels being measured as an indicator of toxicity (Table 3). This problem was overcome by increasing the plating density from 5×10^4 cells to 2×10^5 cells per uncoated tissue culture dish. Although the plating efficiency was much lower on uncoated dishes, a sufficient number of cells attached at the higher plating density for use in Cd experiments.

TABLE 3. EFFECTS OF CELL DISSOCIATION METHOD ON ENERGY CHARGE

Cell Dissociation Method	Energy Charge	% A (X) P		
		ATP	ADP	AMP
Scraped	0.65 ± 0.01	38	52	9
Trypsinized	0.51 ± 0.05	25	52	23

Several types of media were tested for $CdSO_4$ solubility and buffering capacity. A pH indicator (phenol red) was present in some of the media tested and was used to monitor pH changes visually. After adding $CdSO_4$ (final concentration 10 mg/mL), and incubating for 1 h at 37°C and 5% CO_2, a heavy precipitate was formed in Joklik's modified Eagle medium (JMEM), and a slight precipitate was observed in Ham's F12. No precipitate was observed in PBS, Hank's balanced salts solution (Hank's-BSS), or in Ca, Mg-free Hank's medium (CMF-Hank's) (Table 4). The presence and amount of precipitate appeared to correlate with the amount of $NaHCO_3$ present in the different media. In addition, $CdSO_4$ (10 mg/mL) appeared to decrease the pH of JMEM, Ham's F12, and CMF-Hank's.

TABLE 4. SELECTION OF $CdSO_4$ EXPOSURE MEDIUM[a]

Media	Precipitate Formation	pH Indication
JMEM	Heavy	Acidic
Ham's F-12	Slight	Acidic
Hank's-BSS	None	ND[b]
Ca++, Mg++-free HBSS	None	ND
PBS	None	Acidic

a $CdSO_4$ was added to each type of medium to a final concentration of 10 mg/mL. The media were equilibrated in 37°C, 5% CO_2 incubator for 1 h before determining precipitate formation.

b ND-Not determined.

These results suggested that at this high $CdSO_4$ concentration (10 mg/mL), $NaHCO_3$ was not a suitable buffer and may have complexed with the Cd^{2+} to form an insoluble $Cd(CO_3)_2$ precipitate. However, at lower $CdSO_4$ concentrations (1.0 mg/mL), this did not appear to be a problem. Precipitate formation also was not a problem in media containing low $NaHCO_3$ concentrations.

The effects of a range of $CdSO_4$ concentrations on the pH of Ham's F12 are shown in Table 5. After the addition of $CdSO_4$, the media were equilibrated at 37°C and 5% CO_2 for 1.5 h, and the pH measured with a digital pH meter. Physiologically acceptable pH changes occurred after the addition of up to 1.0 mg $CdSO_4$/mL.

TABLE 5. EFFECTS OF $CdSO_4$ CONCENTRATION OF HAM's F-12 MEDIUM

$CdSO_4$ (mg/mL)	pH[a]
0	7.57
0.01	7.55
0.1	7.53
1.0	7.20
10.0	6.83

a After equilibration with $CdSO_4$ for 1 h in a 37°C, 5% CO_2 incubator

In control cultures (no Cd), rinsing with PBS appeared to cause an increase in ATP, AMP, and cAMP levels, and a decrease in nicotinamide adenine diphosphate (NADP) levels relative to non-rinsed controls (Table 6). These changes in nucleotide levels may be a cellular response to environmental changes (temperature, osmolarity, pH, etc.) that occur during rinsing. Cadmium exposure followed by rinsing caused an 80% decrease in ATP levels, while Cd exposure alone caused only a 30% decrease in ATP levels. The ability to increase ATP levels in response to rinsing appeared to be lost by cells exposed to Cd.

TABLE 6. EFFECTS OF CdSO4 EXPOSURE FOLLOWED BY RINSING ON CELLULAR NUCLEOTIDE LEVELS

$CdSO_4$ (mg/mL)	Group		Nucleotides (µg/mL $HClO_4$)					
			ATP	ADP	NADP	AMP	NAD	cAMP
0	No Rinse	Mean	4.71	8.03	8.16	4.95	2.30	1.73
		SD	1.26	1.41	1.64	.63	.11	.43
0.5	No Rinse	Mean	3.31	2.49	3.28	9.47	.42	.85
		SD	.97	.65	.85	2.48	.11	.29
0	Rinsed	Mean	15.71	10.32	2.69	32.47	3.17	2.88
		SD	2.56	2.22	.79	5.60	.41	.24
0.5	Rinsed	Mean	2.91	2.30	2.48	21.44	.36	2.37
		SD	.56	.91	.74	2.72	.00	.42

These effects of Cd and rinsing on ATP, ADP, and AMP levels were evaluated by calculating EC values for each culture. In this experiment, EC values were below those generally measured in human NTE cells (0.70 - 0.80) (Table 7). These relatively low EC values may have been due to the cultures approaching confluence and a state of decreased growth at the time of the experiment.

TABLE 7. EFFECTS OF CdSO4 EXPOSURE FOLLOWED BY RINSING ON CELLULAR ENERGY CHARGE

$CdSO_4$ (mg/mL)	Group	Energy Charge	% Decrease
0	No Rinse	0.490 ± 0.21[a]	–
0.5	No Rinse	0.298 ± 0.008	39
0	Rinsed	0.357 ± 0.017	–
0.5	Rinsed	0.151 ± 0.015	58

[a] Values represent means ± SD, n = 3 cultures.

Rinsing appeared to lower the EC of unexposed cells from 0.490 to 0.357. This difference in EC values was primarily due to the large increase in AMP levels caused by rinsing. Exposure to Cd decreased the EC of both rinsed and non-rinsed cultures. Although Cd exposure lowered the EC by 39%, Cd exposure followed by rinsing resulted in a 58% decrease in EC.

Nucleotide Analyses

Chromatograms of the standard nucleotide solution and of a control human NTE cell extract are shown in Figure 4. The nucleotide concentrations were varied from 50 to 100 ng/mL. Separation was achieved for all nucleotides in the standard solution and for all adenine nucleotides in the control rat NTE cell extracts. Guanine nucleotides also can be analyzed in the cell extracts by varying the buffer pH and CH_3CN content; however, the procedure used in these experiments was optimized for rapid separation of only adenine nucleotides. This procedure did not allow a high resolution of guanine nucleotides. Optimal resolution of adenine nucleotides was obtained when using a

C18 column with high carbon coverage. Separation of nucleotides was highly dependent upon the pH and CH_3CN concentration in buffer A.

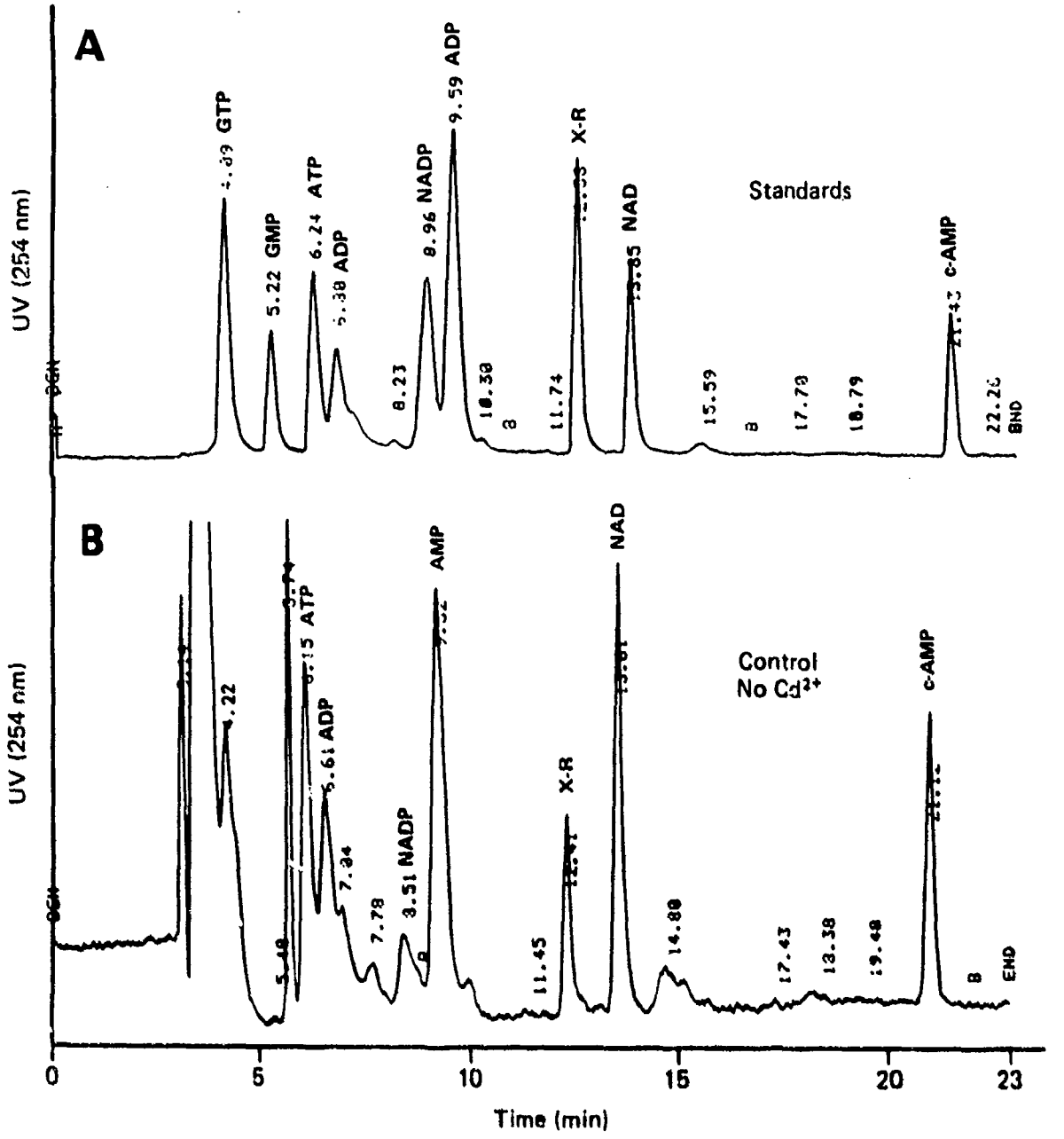

Figure 4. Typical High Performance Liquid Chromatograms of Nucleotides. A. Standards, B. Control NTE cells (no Cd^{2+}).

Figure 5 is a chromatogram of nucleotides from $HClO_4$ extracts of human NTE cells exposed *in vitro* to 0.1, 1, and 10 mg $CdSO_4$/mL for 2 h. The levels of ATP, ADP, and NAD were observed to decrease with increasing $CdSO_4$ concentration, while AMP and NADP levels increased. In addition, an unknown peak was detected after exposure to 1 mg $CdSO_4$/mL and 10 mg $CdSO_4$/mL (retention time = 19.65 min).

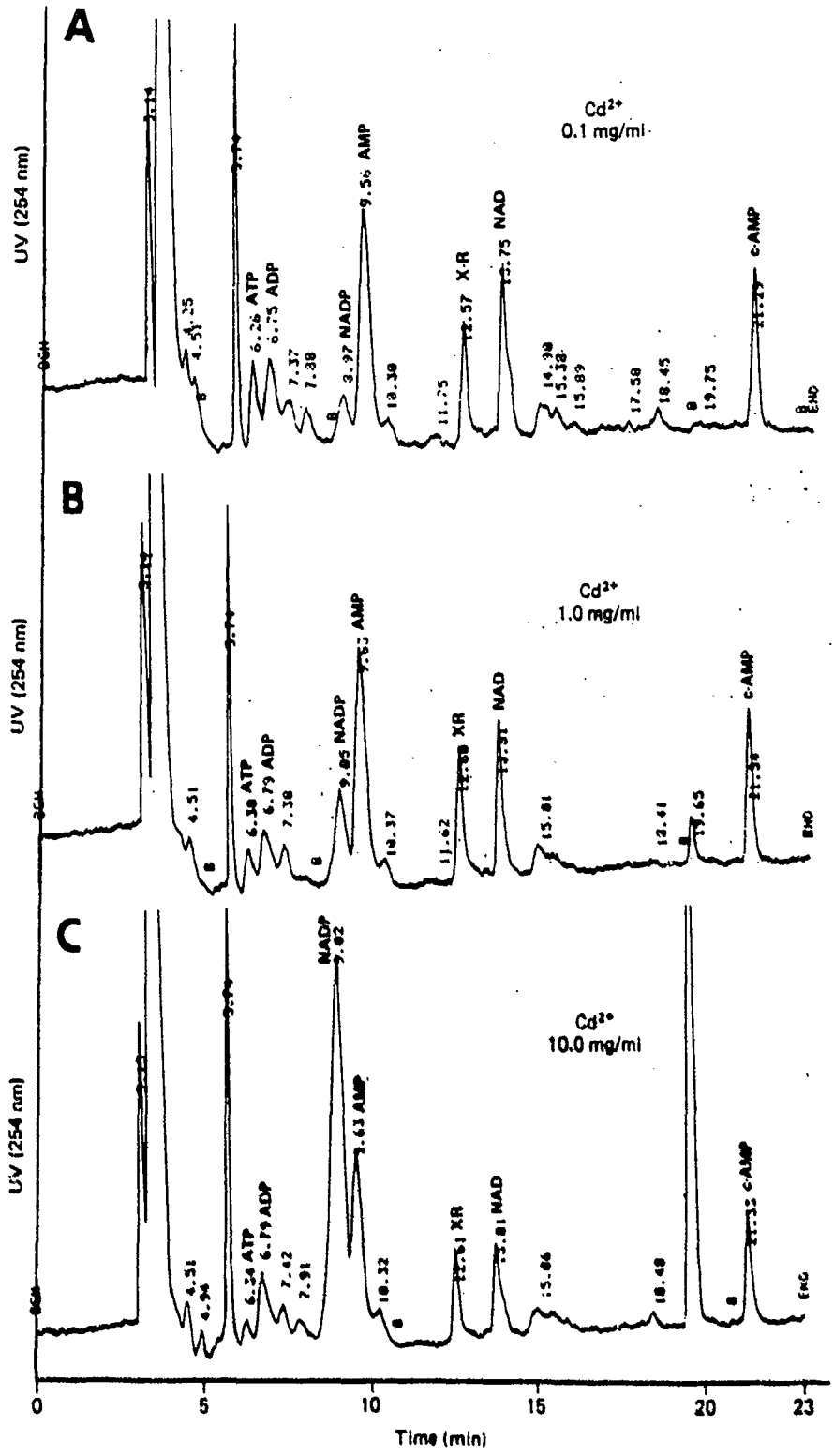

Figure 5. **Typical High Performance Liquid Chromatograms of Nucleotides from $CdSO_4$-Exposed Rat Nasal Turbinate Epithelial Cells.** A. Cells exposed to 0.1 mg/mL Cd^{2+}, B. Cells exposed to 1.0 mg/mL Cd^{2+}, C. Cells exposed to 10mg/ml Cd^{2+}.

Cadmium Uptake by NTE Cells *In Vitro*

To examine possible rat - human differences in the rates of Cd uptake, rat and human NTE cells were exposed to 4.8 mM $CdSO_4$ for up to 240 min. Rat NTE cells took up far less Cd than human NTE cells when exposed to equal concentrations in medium (Table 8). Rat NTE cells attained about 80% of peak levels within 5 min of exposure, while human NTE-cells accumulated only about 45% of peak cellular Cd levels within 5 min of exposure. However, the final Cd levels achieved for rat NTE-cells was only 10-20% that of human NTE cells.

TABLE 8. EFFECTS OF $CdSO_4$ EXPOSURE TIME ON CADMIUM UPTAKE BY RAT AND HUMAN NASAL TURBINATE EPITHELIAL CELLS *IN VITRO*

$CdSO_4$ Exposure Time (min)[a]	Cadium Uptake (µg Cd/µg DNA)	
	Rat	Human
0	0.62 ± .04[b]	0.06 ± .005
5	110 ± 40	534 ± 73
30	115 ± 66	1,086 ± 69
60	122 ± 5	1,108 ± 154
120	149 ± 21	1,046 ± 234
180	131 ± 4	1,245 ± 200
240	145 ± 10	1,207 ± 30

a 1.0 mg $CdSO_4$/mL.
b Mean ± SD, n = 3 cultures.

To explore dose-effects of Cd uptake, rat and human NTE cells were exposed to a range of $CdSO_4$ concentrations for 2 h (Table 9). The amount of Cd uptake after 2 h was similar for human and rat NTE cells exposed to .01 and 0.1 mg/mL. As in the last experiment (see Table 8), after exposure to 1.0 mg/mL $CdSO_4$, a four- to sevenfold greater accumulation of Cd was seen in human cells compared to rat cells.

TABLE 9. EFFECTS OF $CdSO_4$ CONCENTRATION ON CADMIUM UPTAKE BY RAT AND HUMAN NASAL TURBINATE EPITHELIAL CELLS IN VITRO

$CdSO_4$ Concentration (mg/mL)	Cadium Uptake[a] (µg Cd/µg DNA)	
	Rat	Human
0	0.01 ± .003[b]	0.05 ± .012
0.010	0.18 ± 06[c]	0.68 ± .07[c]
0.10	48.7 ± 4.9[c]	43.4 ± 5.1[c]
1.00	250 ± 56[c]	1,057 ± 267[c]

a 2 h exposure time.
b Mean ± SD, n = 3 cultures.
c Significantly different than control ($p < .05$) using analysis of variance.

Nucleotide Levels After *In Vitro* Cd Exposure

Adenine nucleotide levels were measured in human and rat NTE cells after exposure to 1.0 mg/mL $CdSO_4$ for 0 to 4 h to examine species differences (Table 10). Cellular nucleotide levels, expressed as nanograms per microgram DNA, were similar in control cultures of both human and rat NTE cells. In both cell types, a general decrease in ATP, ADP, and NAD levels was observed with increasing exposure time. Levels of ATP decreased to about 56% and 25% of controls in rat and human NTE cells, respectively, with exposure to $CdSO_4$. The ATP and ADP levels increased at the initial time points and then decreased continuously with exposure time to about 50% of control levels in both species. Human cells appeared to have about twofold higher levels of NAD than rat cells, whereas NADP and cAMP levels were approximately the same. Human cell NADP levels increased significantly with exposure time, whereas rat cell NADP levels were changed only at some time points. The AMP levels increased with exposure time, and cAMP levels did not change significantly at any time point in either cell type.

Dose-response relationships were determined by exposure to $CdSO_4$ concentrations ranging from .01 to 0.1 mg/mL for 2 h (Table 11). Not all nucleotides exhibited changes related to the Cd exposure. Both human and rat cells exhibited decreased ATP, ADP, and NAD levels, and increased AMP levels with increasing $CdSO_4$ concentration. ADP and NAD levels expressed per DNA were often two to three times higher in the human cells than in rat cells.

Energy Charge of NTE Cells Exposed *In Vitro*

Cellular concentrations of ATP, ADP, and AMP were used to calculate the EC of rat and human cells after various exposure times (Table 12). Energy charge decreased in both cell types with increasing exposure time. Changes in the EC of human and rat cells in response to $CdSO_4$ exposure were similar, except after long exposure times. After exposure for 240 min, the mean human cell EC was significantly lower than the EC of rat NTE cells ($p<0.05$). These data were similar to the data in Table 10, where ATP levels in human cells were much lower than in rat cells after 240 min of exposure.

The levels of the ATP, ADP, and AMP that were used to calculate EC are shown in Figure 6 for rat and Figure 7 for human NTE cells. Values were expressed as percent of the total adenine nucleotides [%A(X)P]; that is, ATP + ADP + AMP. In both cell types, exposure time appeared to cause a decrease in the percent of ATP and an increase in the percent of AMP with little change in the percent of ADP. The decrease in the percent of ATP was greater in the human cells than in rat cells after exposure for 120 min and longer. The effects of $CdSO_4$ concentration on ATP, ADP, and AMP expressed as percent of A(X)P are shown in Figure 8. In general, the proportion of A(X)P that existed as ATP was lower in human cells. In the rat cells, Cd appeared to cause primarily an increase in the percent of AMP and a decrease in the percent of ATP, whereas in the human cells, Cd also caused an increase in the percent of AMP, but a decrease in both the percent of ATP and percent of ADP.

TABLE 10. EFFECTS OF $CDSO_4$ EXPOSURE TIME[a] ON RAT AND HUMAN NASAL TURBINATE EPITHELIAL CELL NUCLEOTIDE LEVELS[b]

	$CdSO_4$ Exposure Time in Min						
	0	5	30	60	120	180	240
Rat NTE Cells							
ATP	3.07 ± .43[c]	3.64 ± .70	4.38 ± 2.02	3.12 ± 1.39	2.27 ± .51	2.12 ± .16	1.72 ± .14
ADP	0.82 ± .18	0.85 ± .18	0.97 ± .21	0.80 ± .30	0.51 ± .12	0.41 ± .05	0.40 ± .41
AMP	0.36 ± .08	1.36 ± .53	1.87 ± .83[d]	1.76 ± .63[d]	1.45 ± .40[d]	1.68 ± .34[d]	1.84 ± .34[d]
NAD	0.64 ± .04	0.61 ± .18	0.72 ± .42	0.54 ± .18	0.24 ± .05	0.22 ± .06	0.22 ± .12
NADP	0.12 ± .06	0.20 ± .11	0.28 ± .07	0.36 ± .18	0.18 ± .04	0.16 ± .01	0.24 ± .23
cAMP	0.18 ± .10	0.22 ± .03	0.19 ± .06	0.20 ± .13	0.18 ± .10	0.13 ± .01	0.14 ± .04
Human NTE Cells							
ATP	2.20 ± 1.47	2.95 ± 1.22	2.44 ± .68	1.98 ± 1.01	0.86 ± .58	0.48 ± .22	0.58 ± .43
ADP	0.52 ± .44	1.23 ± .42	1.08 ± .33	0.83 ± .45	0.56 ± .48	0.27 ± .07	0.46 ± .29
AMP	0.41 ± .28	0.61 ± .24	0.78 ± .13	0.71 ± .26	0.74 ± .22	0.76 ± .17	0.82 ± .20
NAD	1.08 ± .72	1.62 ± .51	1.36 ± .32	1.25 ± .60	0.96 ± .63	0.67 ± .19	0.79 ± .21
NADP	0.27 ± .21	1.09 ± .23	1.58 ± .29	2.17 ± 1.03[d]	2.02 ± 1.09[d]	2.23 ± .57[d]	2.36 ± .72[d]
cAMP	0.27 ± .20	0.52 ± .33	0.32 ± .17	0.33 ± .08	0.45 ± .31	0.32 ± .22	0.36 ± .05

[a] 1.0 mg CdSO4/mL.
[b] µg Nucleotide/µg DNA.
[c] Values represent means ± SD, n = 3 cultures.
[d] Significantly different ($p < .05$) from control (0 time).

TABLE 11. EFFECTS OF $CDSO_4$ CONCENTRATION[a] ON NUCLEOTIDE LEVELS[b] OF RAT AND HUMAN NASAL TURBINATE EPITHELIAL CELLS

	$CdSO_4$ Exposure (mg/mL)			
	0	0.01	0.1	1.0
Rat NTE Cells				
ATP	3.20 ± .43[c]	1.70 ± .34[d]	1.67 ± .09[d]	0.93 ± .14[d]
ADP	0.82 ± .32	0.47 ± .16	0.61 ± .05	0.42 ± .05
AMP	0.91 ± .31	0.42 ± .09	0.76 ± .24	1.40 ± .23
NAD	0.53 ± .40	0.38 ± .10	0.39 ± .04	0.23 ± .09
NADP	0.2 ± .13	0.08 ± .02	0.18 ± .01	0.10 ± .04
cAMP	0.19 ± .05	0.16 ± .14	0.20 ± .02	0.09 ± .05
Human NTE Cells				
ATP	2.93 ± .64	3.03 ± .46	1.41 ± 1.16	2.73 ± .74
ADP	3.24 ± 1.02	2.24 ± .68	0.79 ± .83	1.14 ± .45
AMP	1.11 ± .44	1.18 ± .14	1.18 ± .15	7.95 ± 1.67[d]
NAD	1.53 ± .56	1.53 ± .25	0.75 ± .35	0.79 ± .26
NADP	0.32 ± .16	0.25 ± .03	0.23 ± .08	0.11 ± .04
cAMP	0.44 ± .04	0.33 ± .33	0.34 ± .06	0.32 ± .11

[a] 2-h exposure.
[b] µg nucleotide/µg DNA.
[c] Values represent means ± SD, n = 3 cultures.
[d] Significantly different ($p < .05$) from control (0 time).

TABLE 12. EFFECTS OF $CdSO_4$ EXPOSURE TIME ON THE ENERGY CHARGE OF RAT AND HUMAN NASAL TURBINATE EPITHELIAL CELLS EXPOSED *IN VITRO*

	Energy Charge[a] Exposure Time (min)[b]						
	0	5	30	60	120	180	240
Human	.79 ± .01	.74 ± .01	.69 ± .53	.67 ± .04	.50 ± .07[c]	.45 ± .13[c]	.30 ± .02[d]
Rat	.81 ± .02	.70 ± .03[c]	.67 ± .01[c]	.62 ± .04[c]	.60 ± .03[c]	.55 ± .03[c]	.49 ± .05[c]

[a] Exposed to 1.0 mg $CdSO_4$/mL
[b] Values represent means ± SD, n = 3 cultures
[c] Significantly different ($p < 0.05$) from control (0 time)
[d] Significantly different from rat cell energy charge after the same exposure time ($p < 0.05$).

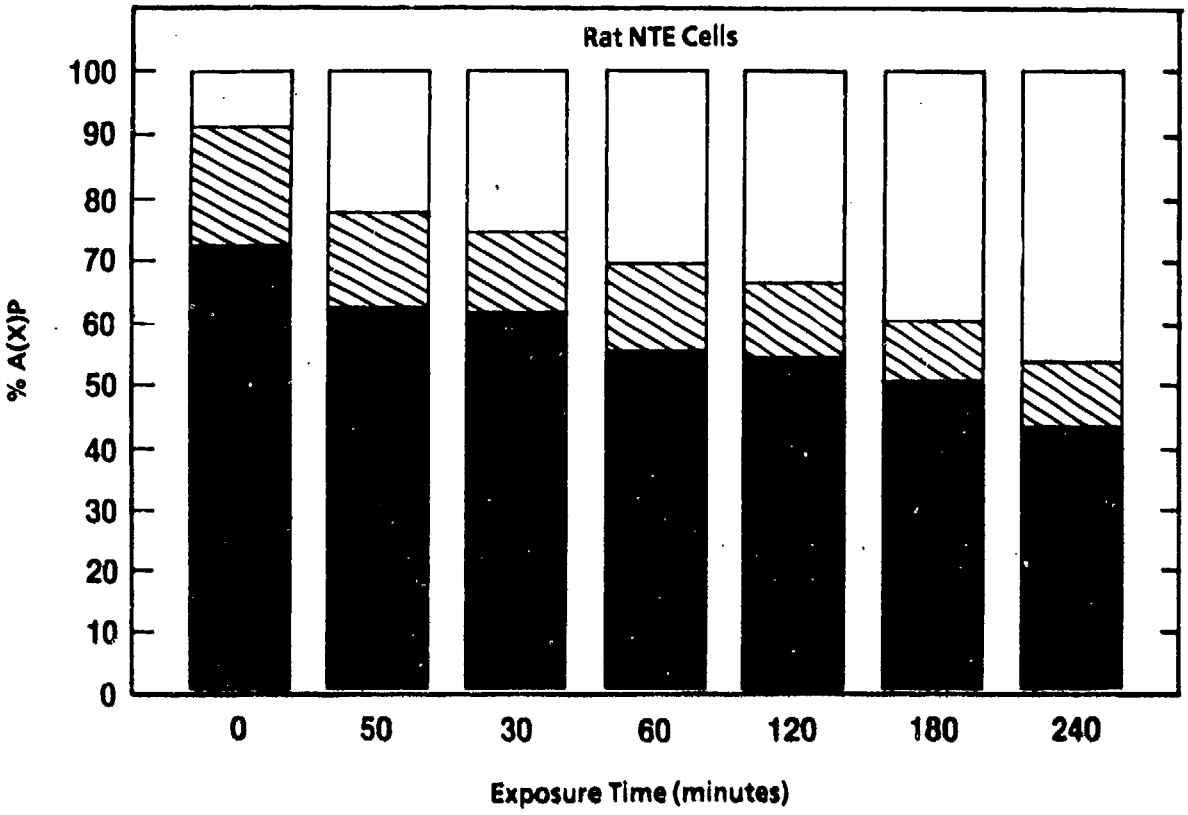

Figure 6. Effects of $CdSO_4$ Exposure Time on Rat Nasal Turbinate Epithelial Cell Adenine Nucleotide Levels. □, AMP; ▨, ADP; ■, ATP.

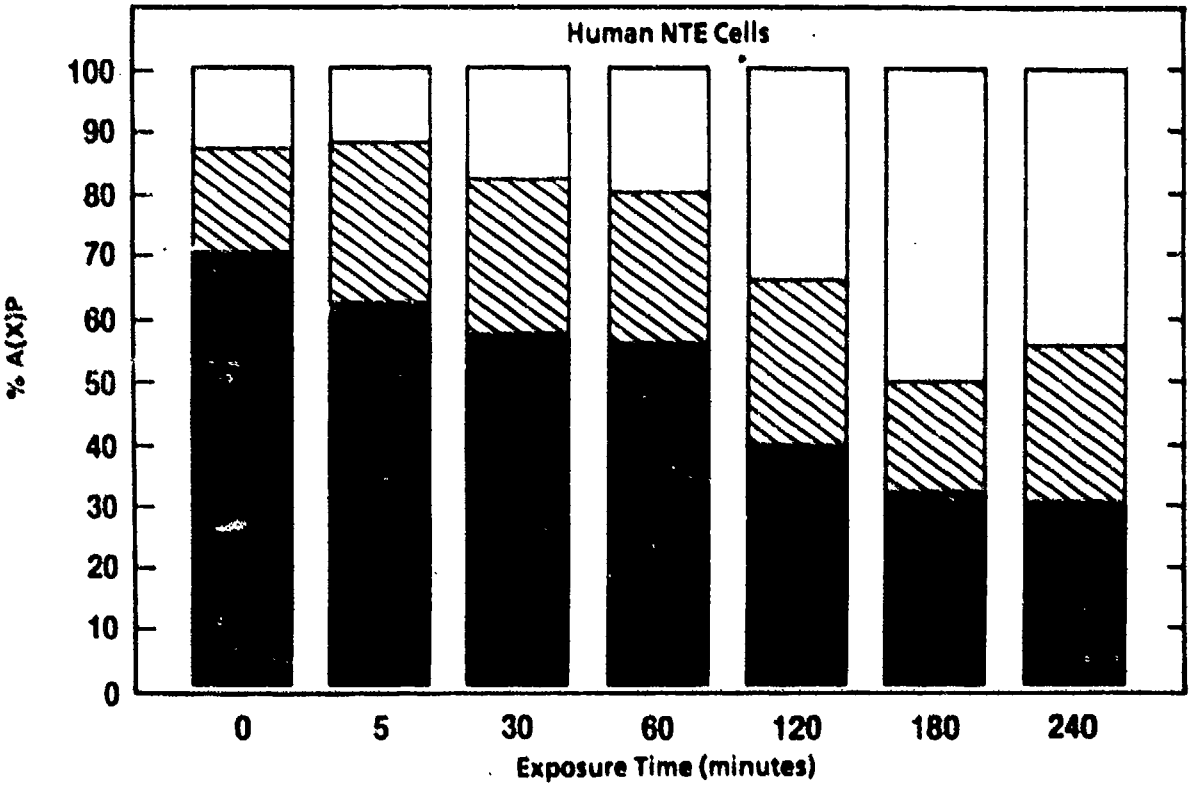

Figure 7. Effects of $CdSO_4$ Exposure Time on Human Nasal Turbinate Epithelial Cell Adenine Nucleotide Levels. □, AMP; ▨, ADP; ■, ATP.

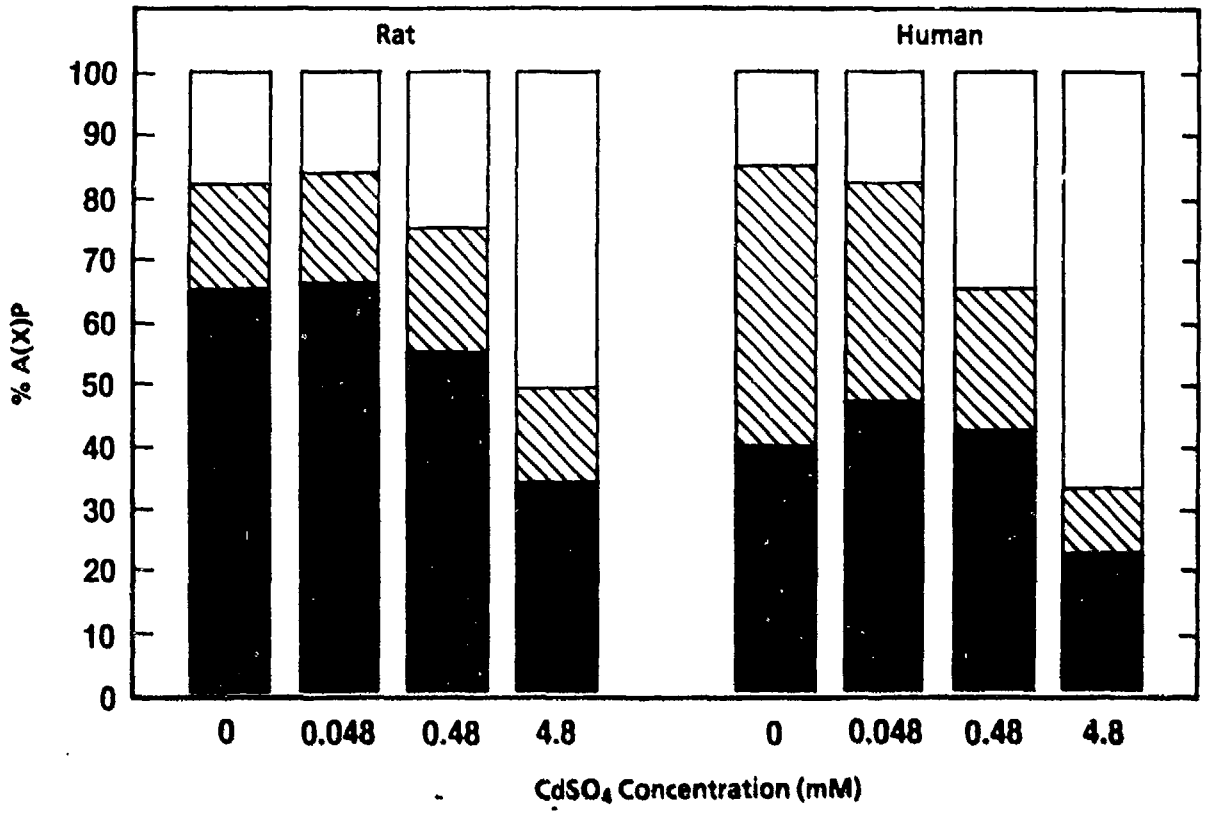

Figure 8. Effects of $CdSO_4$ Exposure Concentration on Adenine Nucleotide Levels in Rat and Human Nasal Turbinate Epithelial Cells Following a 2-h Exposure. □, AMP; ▨, ADP; ■, ATP.

The effects of $CdSO_4$ concentration on the EC of human and rat NTE cells were also investigated (Table 13). Because control values were slightly lower for human cells in this experiment, data were expressed as percent control values. A concentration dependent decrease in EC was observed for both human and rat NTE cells, with no significant differences after exposure to 0.01 mg/mL. However, at $CdSO_4$ concentrations of 0.1 and 1 mg/mL, the EC of human cells was decreased significantly more than that of the rat NTE cells.

TABLE 13. EFFECTS OF $CdSO_4$ CONCENTRATION ON THE ENERGY CHARGE OF HUMAN AND RAT NASAL TURBINATE EPITHELIAL CELLS

$CdSO_4$ Concentration (mg/mL)[b]	Energy Charge (% Control)[a]			
	Human		Rat	
0	0.63 ± .03	(100 ± 4.4)	0.73 ± .06	(100 ± 6.7)
0.01	0.64 ± .02	(102 ± 2.7)	0.75 ± .01	(103 ± 1.5)
0.10	0.48 ± .17	(76 ± 26.4)[c]	0.65 ± .04	(89 ± 4.8)[c,d]
1.00	.28 ± .02	(44 ± 2.5)[c]	0.42 ± 0.03	(57 ± 2.9)[c,d]

[a] The control energy charge values were 0.63 ± 0.02 for human cells and 0.73 ± 0.05 for rat cells. Values are means ± SD, n = 3 cultures.
[b] *In vitro* exposure for 2 h.
[c] Significantly different than corresponding control ($p < 0.05$).
[d] Significantly different than human cell energy charge at the same exposure concentration ($p < 0.05$).

Alkaline Elution

In an initial experiment, rat NTE cells were used to test the alkaline elution procedure. Two aliquots of untreated cells were placed on the columns and eluted under alkaline conditions (Figure 9). Calf thymus DNA was used to generate a standard curve (Figure 10) for quantifying the DNA using a microfluorometric technique. DNA from untreated control cells usually passed through the filter very slowly, and in this assay, about 62% remained on the filter after seven 90-min fractions had been collected.

To measure the ability of this assay to detect DNA strand breaks, rat NTE cells were exposed to concentrations of Cd known to have DNA damaging effects (Figure 11). Both untreated NTE cells and NTE cells that had been exposed to 1 mg $CdSO_4$ /mL for 2 h were analyzed. The control DNA eluted to 73% in 5.5 h, while the DNA from the Cd-exposed cells eluted faster and only 50% remained after 5.5 h. Major differences in elution rates were seen in the first 90-min fraction.

In the third experiment, the ability of the assay to provide dose response data was assessed. Rat NTE cells were exposed to 0, 0.05, 0.5, and 5 mg $CdSO_4$ /mL for 2 h, and then eluted as before (Figure 12). The untreated control DNA eluted to 53% in 5.5 h, and the DNA from the 0.05 and 0.5 mg $CdSO_4$ /mL eluted to 15 and 17%, respectively, and the DNA from the 5 mg/mL group eluted to 37%. The lower elution rate of the 5 mg/mL group may have been an artifact due to the high dose.

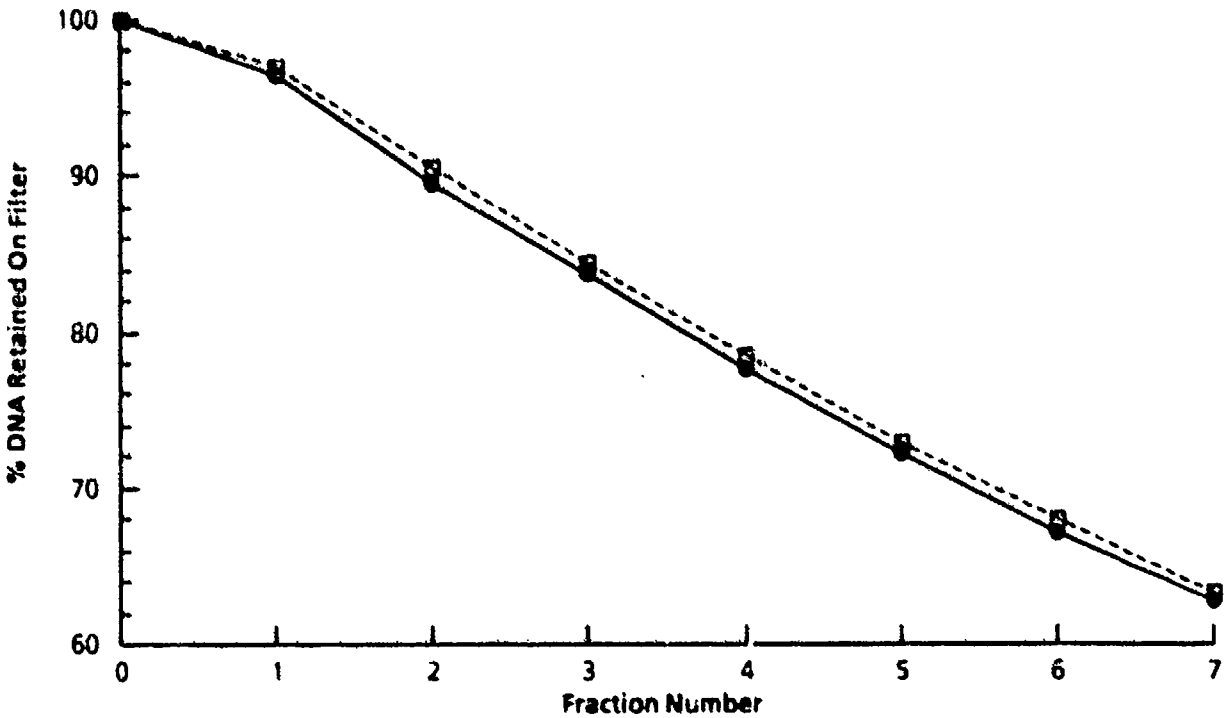

Figure 9. Alkaline Elution Assay of DNA from Control Rat Nasal Turbinate Epithelial Cells. ●, 9.25×10^6 cells; □, 7×10^6 cells.

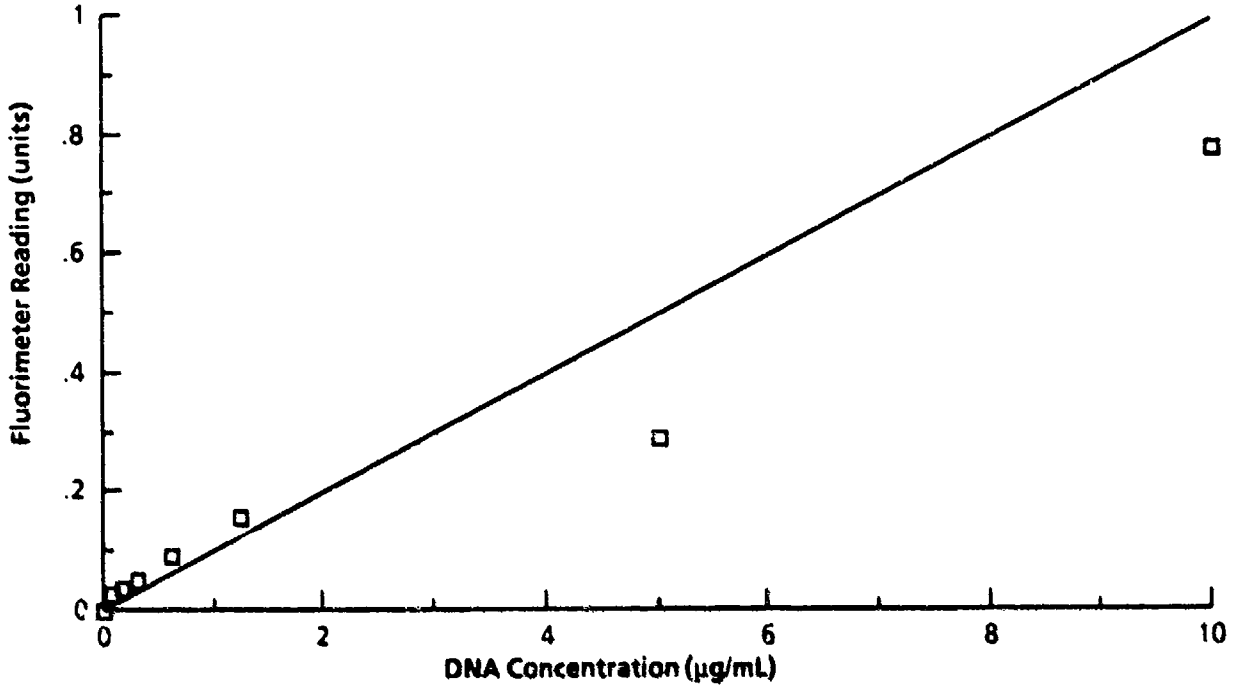

Figure 10. DNA Standard Curve Using Calf Thymus DNA for Alkaline Elution Assay. □, Actual readings; ——, regression line.

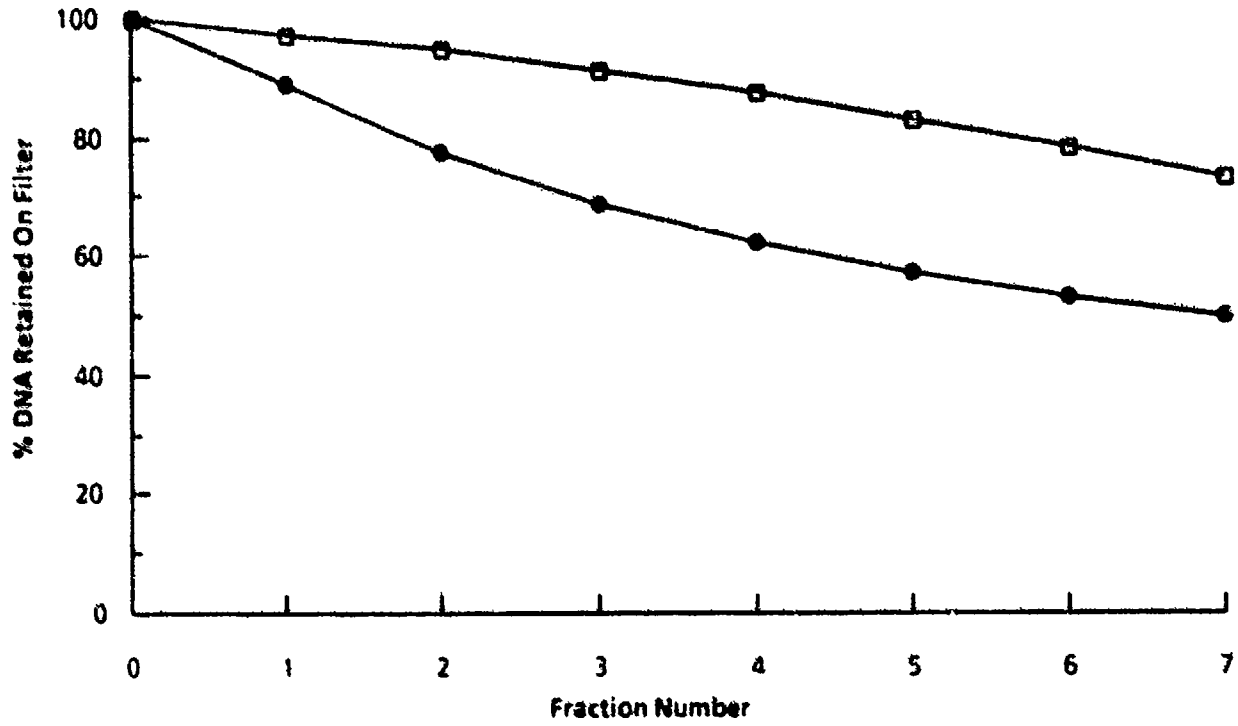

Figure 11. Alkaline Elution Assay of DNA from Rat Nasal Turbinate Epithelial Cells Exposed *In Vitro* to $CdSO_4$. □, Control; ●, 1 mg/mL.

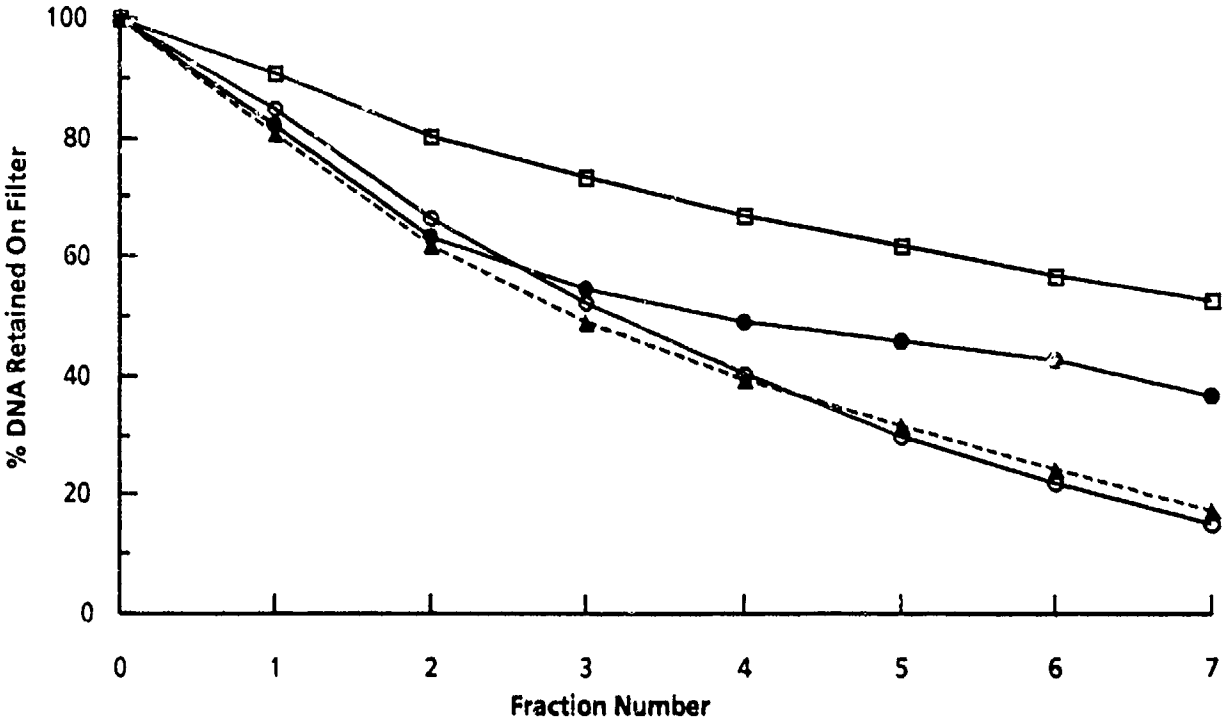

Figure 12. Alkaline Elution Assay of DNA from Rat Nasal Turbinate Epithelial Cells Exposed *In Vitro* to $CdSO_4$. □, Control; O, 0.05 mg/mL; ▲, 0.5 mg/mL; ●, 5 mg/mL.

IN VIVO NASAL EPITHELIUM

In dose-response studies, rats were exposed for 2 h by nose-only to 0, 5, or 10 mg $CdSO_4/m^3$, and the nasal epithelial tissues were analyzed for Cd, changes in nucleotides, and EC (Table 14). Exposure to 5 mg/m³ resulted in NTE tissue levels of 2.6 ng/mg wet weight, and doubling the exposure concentration to 10 mg/m³ increased the tissue dose to 3.1 ng Cd/mg wet weight.

TABLE 14. EFFECTS OF $CDSO_4$ AEROSOL EXPOSURE ON RAT NASAL TURBINATE EPITHELIAL TISSUE NUCLEOTIDE LEVELS

Exposure Concentration (mg/m³)[a]	NTE Tissue Cd Levels (ng/mg wet weight)	% Total A(X)P[b]			Energy Charge
		ATP	ADP	AMP	
0	0.07 ± 02	59 ± 4.5	32 ± 3.5	9 ± 1.2	.75 ± .04
5	2.6 ± 53	54 ± 3.2	36 ± 6.3	10 ± 3.1	.72 ± .01
10	3.1 ± 51	54 ± 4.4	33 ± 2.1	15 ± 6.6	.70 ± .04

[a] Rats were exposed to $CdSO_4$ aerosols for 2 h by the nose-only method. Values represent means ± SD, n = 6 rats.
[b] Total A(X)P = ATP + ADP + AMP.

The energy charge of NTE tissues was decreased but not changed significantly by exposure to 5 or 10 mg $CdSO_4/m^3$. Adenosine triphosphate levels (%A(X)P) appeared to decrease, while AMP levels (%A(X)P) increased slightly with increasing $CdSO_4$ concentration. The EC calculated from these

nucleotide data appeared to decrease dose-dependently, although these changes were not statistically significant.

Two additional *in vivo* $CdSO_4$ exposures were performed to verify the slight changes in EC observed in previous studies and to determine if Cd effects on EC were delayed. In these studies, rats (five/group) were exposed to 10 mg $CdSO_4/m^3$ or room air for 2 h, then sacrificed, and another group of animals was sacrificed 4 h after exposure.

Animals used in both experiments were approximately the same age and had similar body weights. The amount of nasal epithelial tissue removed from each animal was relatively consistent between rats and between the two studies (Table 15). The amounts of Cd contained in the exposed tissue samples were slightly higher in Experiment 1 than in Experiment 2 (Table 16).

TABLE 15. NASAL TISSUE WEIGHTS FROM RATS EXPOSED BY INHALATION TO $CdSO_4$

Exposure Concentration (mg/m^3)[a]	Hours After Exposure	Nasal Tissue Weights (mg)	
		Expt. 1	Expt. 2
0	0	51.2 ± 8.6	39.3 ± 6.7
10	0	45.2 ± 11.8	44.7 ± 6.2
0	4	41.7 ± 5.8	47.5 ± 4.2
10	4	41.1 ± 16.9	40.9 ± 7.6

[a] Rats were exposed to $CdSO_4$ for 2 h by the nose-only method.

TABLE 16. NASAL TISSUE CADMIUM LEVELS FOLLOWING INHALATION EXPOSURE TO CdSO4

Exposure Concentration (mg/m^3)[a]	Hours After Exposure	ng Cd/mg Wet Weight (mg)	
		Expt. 1	Expt. 2
0	0	0.043 ± 0.008	0.054 ± 0.008
10	0	5.302 ± 1.201	2.904 ± 1.147
0	4	.055 ± 0.012	.053 ± 0.019
10	4	5.706 ± 2.524	3.306 ± 1.328

[a] Rats were exposed to $CdSO_4$ for 2 h by the nose-only method.

Exposure to Cd appeared to decrease the EC in both experiments when measured at 0 or 4 h after exposure (Table 17). In Experiment 1, the largest decrease was observed immediately after exposure, whereas in Experiment 2, the largest decrease was detected 4 h after exposure. The decrease in EC in both studies was due to decreased levels of ATP with increases in ADP and AMP (Figures 13 and 14). Mean values obtained by pooling data from these two experiments are shown in Table 18.

TABLE 17. ENERGY CHARGE MEASUREMENTS OF NASAL TISSUE FROM RATS EXPOSED TO $CdSO_4$

$CdSO_4$ Concentration (mg/m^3)	Hours After Exposure	Expt. 1		Expt. 2	
		Energy Charge	% Decrease	Energy Charge	% Decrease
0	0	.701 ± .004	–	.727 ± .027	–
10	0	.657 ± .017[a]	6.3	.717 ± .030	1.2
0	4	.687 ± .016	–	.718 ± .009	–
10	4	.662 ± .005[a]	3.6	.687 ± .038	4.3

[a] Significantly different ($p<0.05$) from control (0 concentration).

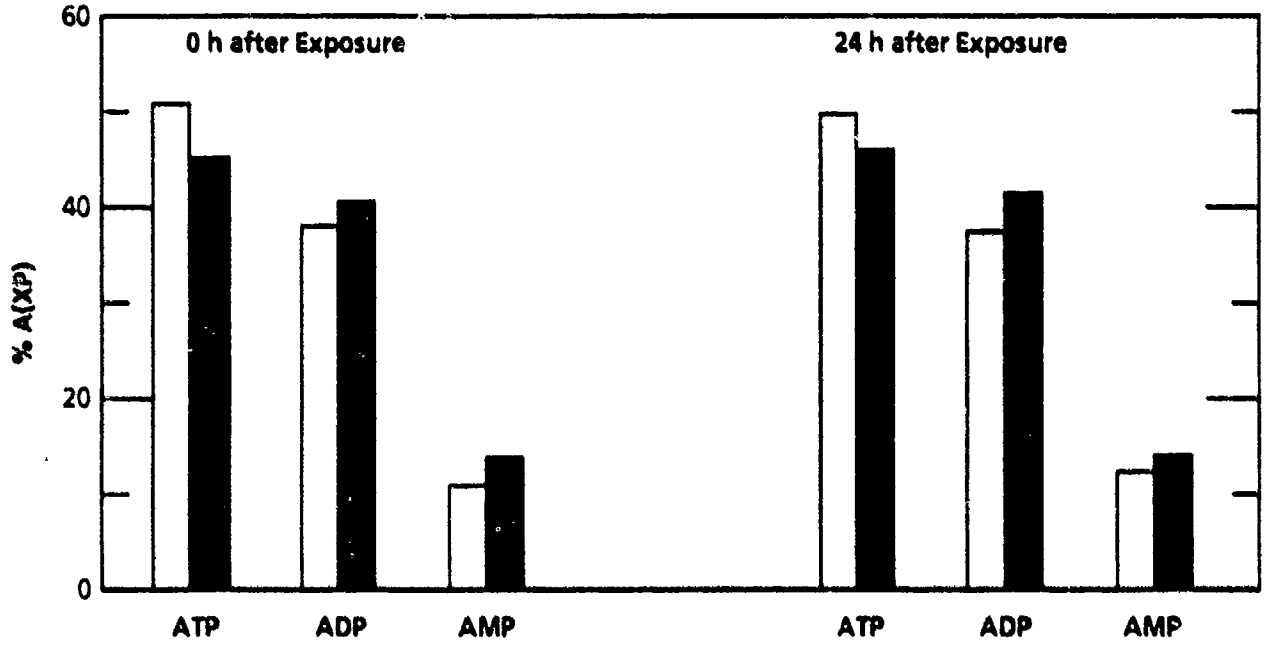

Figure 13. Alkaline Elution Assay of DNA from Rat Nasal Turbinate Epithelial Cells Exposed *In Vitro* to $CdSO_4$. Determinations were made 4 h following the end of a 2-h exposure. Experiment 1. □, 0 mg/m^3; ■, 10 mg/m^3.

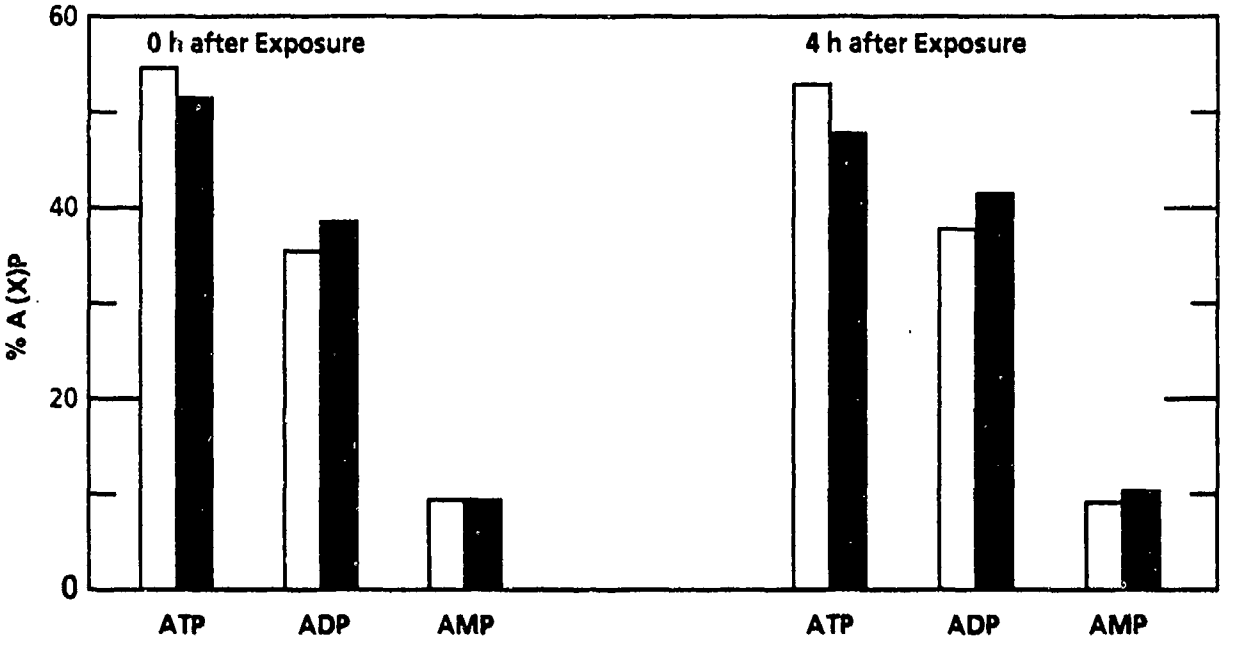

Figure 14. Relative Adenine Nucleotide Levels in Rat Nasal Epithelial Cells Exposed to *In Vivo* $CdSO_4$-Experiment 2. Determinations were made 4 h following the end of a 2-h exposure. Experiment 2. □, 0 mg/m^3; ■, 10 mg/m^3.

TABLE 18. EFFECTS OF $CdSO_4$ INHALATION ON ENERGY CHARGE OF RAT NASAL EPITHELIUM

$CdSO_4$ (mg/m^3)	Hours After Exposure	ng Cd/mg Wet Weight	Energy Charge	% A(X)P[a]		
				ATP	ADP	AMP
0	0	.049 ± .009[b]	.71 ± .02	53	37	10
10	0	4.57 ± 1.46[c]	.69 ± .04	48[c]	40[c]	12
0	4	.054 ± .016	.70 ± .02	51	38	11
10	4	4.29 ± 2.63[c]	.68 ± .03	48	40	12

[a] A(X)P = (ATP + ADP + AMP).
[b] Values represent pooled values from Expt 1 and Expt 2 (Tables 16,17). Means ± SD of at least 10 rats.
[c] Significantly different ($p<0.05$) from control (0 time).

The effects of *in vivo* $CdSO_4$ exposure on glutathione and ascorbic acid (Figure 15) levels in rat nasal epithelium were also measured at 0 and 4 h after exposure. Glutathione and ascorbic acid were slightly decreased immediately after exposure; however, when measured 4 h after exposure, both were increased by about twofold.

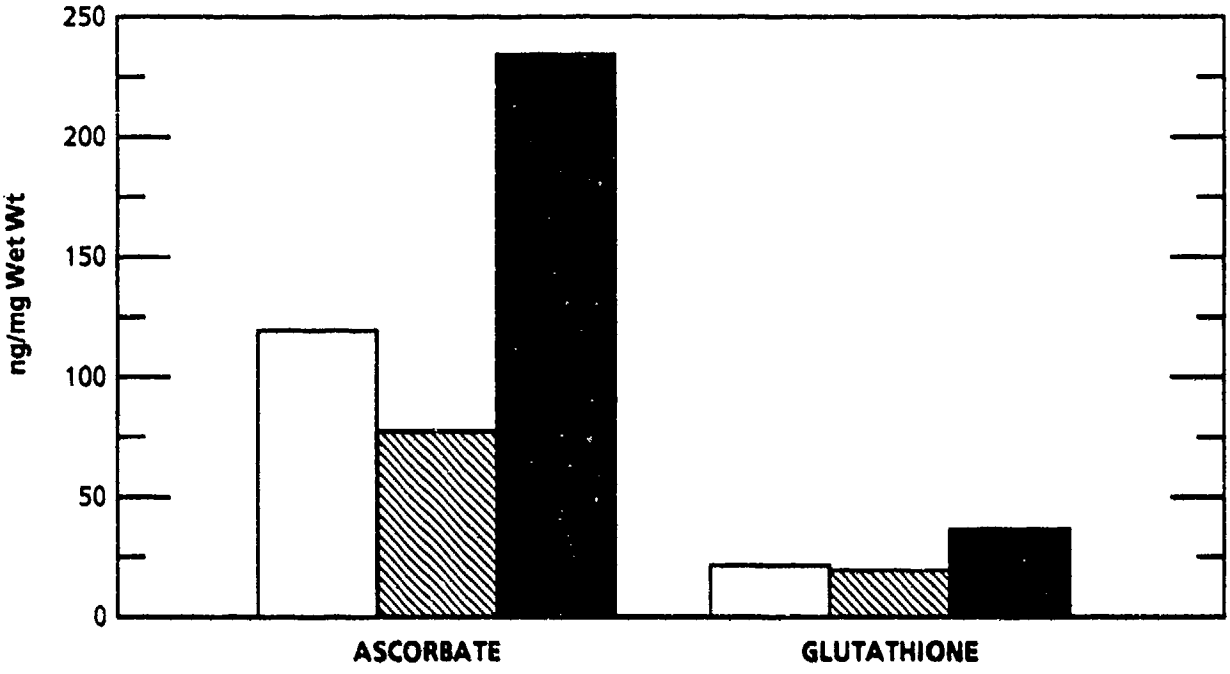

Figure 15. Effects of CdSO4 on Glutathione and Ascorbate Levels in Nasal Tissues from Rats Exposed *In Vivo* to $CdSO_4$. Rats were exposed to 10 mg/m^3 for 2 h. □, Control at 0 h post-exposure; ▨, Cd-exposed at 0 h post-exposed; ■, Cd-exposed at 4 h post-exposure.

Histology

To determine if $CdSO_4$ caused tissue damage that was not detected by EC measurements and if the damage could be visually observed in the respiratory or olfactory regions, a histological evaluation of rat nasal tissue was performed. Rats were exposed to 10 mg $CdSO_4$/m^3 by nose-only inhalation, and sacrificed by exsanguination under anesthesia at 0, 4, and 24 h after exposure. The nasal cavities were immediately perfused with formalin and all excess tissue removed. These samples were prepared and evaluated in cooperation with Dr. Kevin Morgan of the Chemical Industry Institute of Technology, Research Triangle Park, NC. In the respiratory tissue, indicators of Cd-induced damage appeared to be a loss of cilia and in some areas a disruption of the epithelium. However, these indicators of damage were most evident in the olfactory tissue and appeared to be greater in the 24-h group than in the 0- or 4-h groups (Figures 16A and B).

A

B

Figure 16. Rat Nasal Olfactory Epithelium. A. Control rats, B. $CdSO_4$-exposed rats.

To determine if this apparent selective regional toxicity was a result of differences in Cd deposition, respiratory and olfactory nasal tissues were removed at 0, 4, and 24 h after a 2-h exposure to 10 mg/m^3. Olfactory and respiratory nasal epithelium from control rats (no Cd) contained similar background amounts of Cd (Figure 17). Exposure to 10 mg $CdSO_4$/m^3 for 2 h resulted in about a threefold increase in Cd levels of olfactory cells. The Cd levels did not decrease with time after exposure (up to 24 h). Cadmium levels in respiratory epithelium were increased by about eightfold, compared to controls when measured immediately after exposure, and decreased to about threefold with time after exposure. When measured 24 h after exposure, the Cd levels of respiratory epithelium were not significantly different than Cd levels of olfactory epithelium.

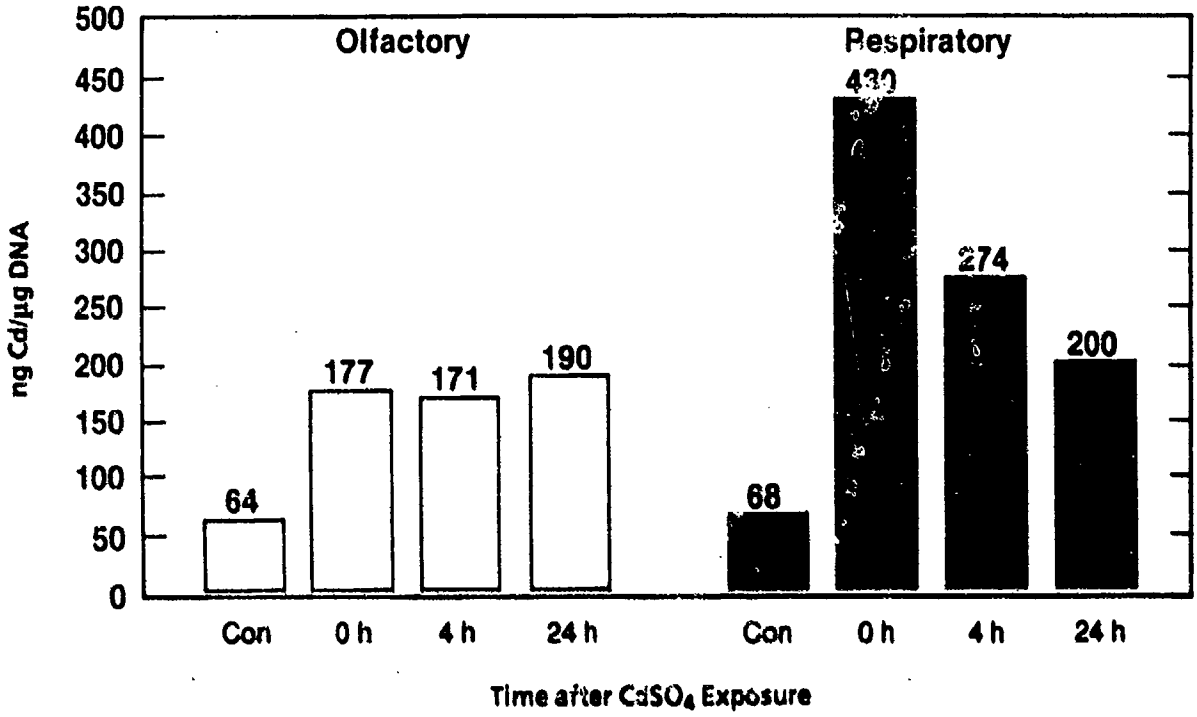

Figure 17. Cadmium Levels in Olfactory and Respiratory Epithelial Tissue from $CdSO_4$-Exposed Rats. Six rats per group were exposed to 10 mg/m^3 for 2 h.

Nucleotides and Energy Charge

Although considerably higher levels of Cd were detected in respiratory epithelium, Cd-induced changes in nucleotide and EC levels were almost identical for respiratory and olfactory epithelium (Figures 18 and 19). For both types of epithelium, ATP and EC levels were lowest immediately after exposure and returned to control levels by 24 h after exposure. The ATP levels in olfactory tissue were about 10 % lower than in respiratory tissue.

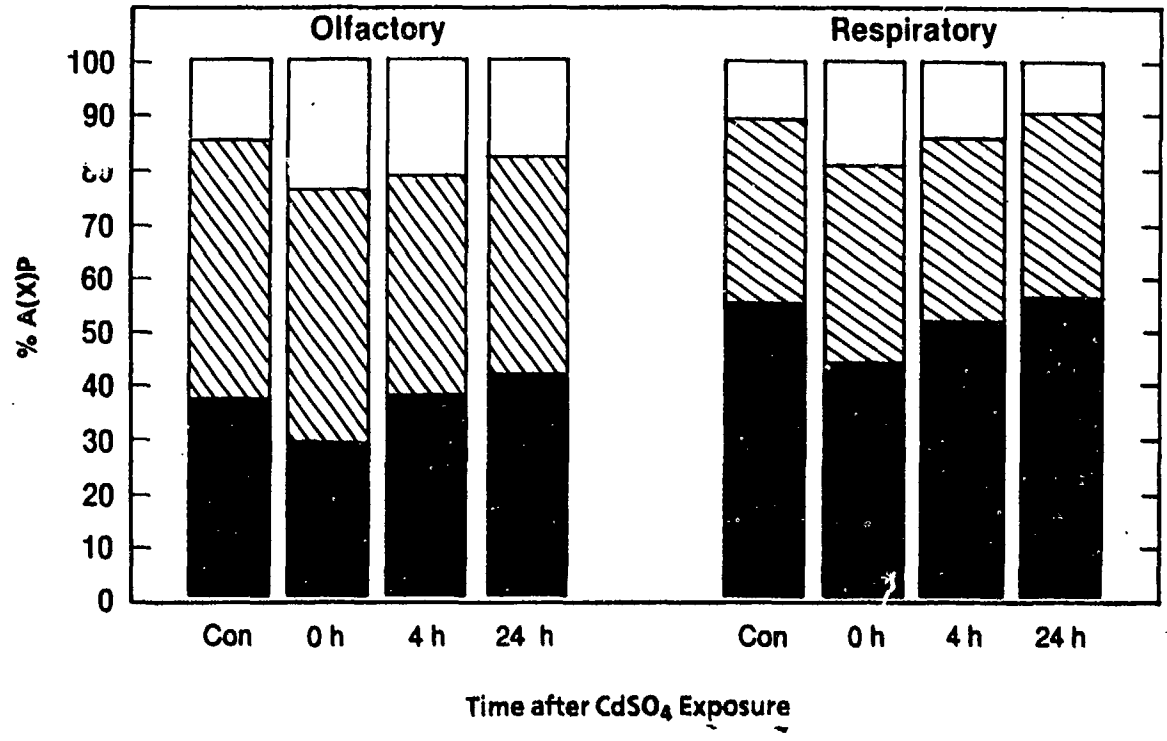

Figure 18. Adenine Nucleotide Levels in Nasal Olfactory and Respiratory Epithelium from $CdSO_4$-Exposed Rats. Six rats per group were exposed to 10 mg/m^3 for 2 h. □, AMP; ▨, ADP; ■, ATP.

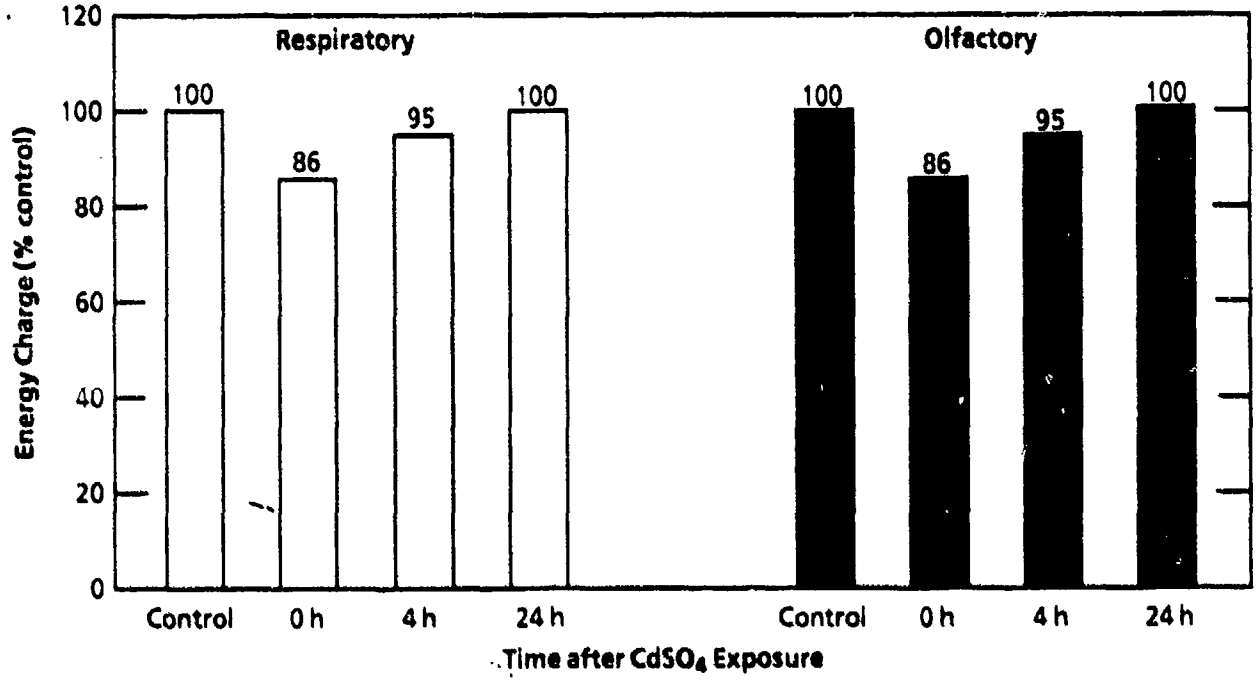

Figure 19. Energy Charge of Olfactory and Respiratory Epithelial Tissue from $CdSO_4$-Exposed Rats. Six rats per group were exposed to 10 mg/m^3 for 2 h.

NASAL LAVAGE FLUID

In an initial experiment to test the nasal lavage procedure, unexposed rats were anesthetized and the trachea cannulated to the nasopharynx. The nasal cavity was then lavaged *in situ* via the tracheal cannula, and the lavage fluid collected at the nostrils.

Data from a preliminary experiment to test the feasibility of performing $HClO_4$ extractions *in situ* indicated that nucleotides could be extracted from the nasal epithelium in as little as 15 s (Table 19). The total amount of nucleotides was 3 to 5 times greater after the two 5-min incubations than after the two 15-s rinses. Although nucleotide levels were expected to increase with extraction time, it is not apparent why the ECs were greater after the longer extraction periods (see Table 20).

TABLE 19. *IN SITU* EXTRACTION OF NUCLEOTIDES BY NASAL LAVAGE

Lavage Procedure	Total Adenine Nucleotides Extracted (µg/mL Lavage Fluid)
PBS Rinse (15 s)	1.1
$HClO_4$ Rinse (15 s)	7.0
$HClO_4$ Rinse (5 min)	18.4

TABLE 20. *IN SITU* EXTRACTION OF RAT NASAL EPITHELIAL NUCLEOTIDES AFTER *IN VIVO* $CdSO_4$ EXPOSURE[a]

	Energy Charge	%A(X)P[b]		
		ATP	ADP	AMP
PBS Rinse				
$CdSO_4$	.64 ± .15[c]	58 ± 11	12 ± 12	30 ± 20
Control	.55 ± .22	34 ± 19	42 ± 14	24 ± 27
$HClO_4$ –15-s Rinse				
$CdSO_4$	.77 ± .09	65 ± 11	24 ± 4	12 ± 7
Control	.77 ± .08	66 ± 10	21 ± 5	13 ± 7
$HClO_4$ –5-min Rinse				
$CdSO_4$	.72 ± .05	61 ± 6	22 ± 5	17 ± 5
Control	.75 ± .03	64 ± 3	22 ± 2	14 ± 2

[a] 10 mg $CdSO_4/m^3$ for 2 h.
[b] A(X)P = ATP + ADP + AMP.
[c] Mean ± SD, n = 4 rats.

This experiment was repeated using rats exposed to 0 and 10 mg $CdSO_4/m^3$ for 2 h. The nasal passages were first rinsed with PBS containing 10 mM EDTA to remove most of the mucus and free Cd. This PBS rinse was followed by a 15-s $HClO_4$ rinse and a 5-min $HClO_4$ incubation to extract the epithelial cell nucleotides. After the 5-min $HClO_4$ incubation, the nose was rinsed with buffer to neutralize the $HClO_4$, and then filled with a pronase/collagenase solution for 15 min to dissociate the epithelial cells. The dissociated cells were collected by flushing the nasal passages with 20 mL of PBS.

Energy charge and nucleotide levels in PBS and $HClO_4$ lavage fluid samples were not significantly different between control and exposed rats (Table 20). The Cd analyses showed significantly higher levels of Cd in exposed rats (Table 21). Most of the Cd (75%) was detected in the mucus lining of the nasal passages, which was removed by the PBS rinse. The $HClO_4$ extracts removed about 24% of the Cd, while only about 1.3% was detected in the cell pellet.

TABLE 21. CADMIUM EXTRACTION BY NASAL LAVAGE OF $CdSO_4$-EXPOSED RATS[a]

Lavage Procedure	Cd Concentration	% Total Cd
PBS – 15 s	(ng/5 mL)	
Cd-exposed	2,688 ± 2,325[b]	75.0
Controls	140 ± 221	
$HClO_4$ – 15 s	(ng/1.5 mL)	
Cd-exposed	83 ± 145	2.3
Controls	3 ± 0.4	
$HClO_4$ – 5 min	(ng/1.5 mL)	
Cd-exposed	770 ± 510	21.5
Controls	9 ± 12	
Nasal Cells	(ng/PELLET)	
Cd-exposed	45 ± 50	1.2
Controls	<1	

[a] Rats were exposed to 10 mg $CdSO_4/m^3$ or room air (controls) for 2 h.
[b] Values represent means ± SD, n = 4 rats.

In situ extraction of nasal epithelium was also performed using a newly designed nasal lavage tube. In this experiment, two groups of rats were exposed to 10 mg $CdSO_4/m^3$ for 2 h and nasal lavages performed 0 and 24 h after exposure. Two control groups were exposed to room air. In PBS lavage fluid, Cd levels remained unchanged over 24 h in exposed rats (Table 22). Cadmium levels appeared to increase by 24 h in $HClO_4$ lavage fluid from exposed rats. Energy charge values were calculated from nucleotide levels detected in PBS and $HClO_4$ rinses. Energy charges were lower for the 24-h PBS rinses than for the 0-h rinses. At 0 and 24 h after exposure, EC values for control PBS rinses were lower than EC values for PBS rinses from exposed rats. Energy charges of $HClO_4$ rinses were about the same for all groups, except for the slightly higher EC of 24-h control rinses.

TABLE 22. CADMIUM LEVELS AND ENERGY CHARGE IN RAT NASAL LAVAGE FLUID OF RATS EXPOSED TO $CdSO_4$

		Cd Levels (µg/Total Rinse)	
	Hours Post-Exposure[a]	PBS Rinse (5 mL)	$HClO_4$ Rinse (3 mL)
Control	0	0.18 ± 0.28[b]	0.012 ± 0
Exposed	0	3.40 ± 0.96	4.5 ± 2.3
Control	24	0.07 ± 0.10	0.012 ± 0
Exposed	24	3.40 ± 2.15	7.0 ± 10.5

		Energy Charge	
	Hours Post-Exposure[a]	PBS Rinse (5 mL)	$HClO_4$ Rinse (3 mL)
Control	0	0.35 ± 0.10	0.56 ± 0.15
Exposed	0	0.62 ± 0.18	0.57 ± 0.19
Control	24	0.26 ± 0.07	0.70 ± 0.08
Exposed	24	0.42 ± 0.09	0.59 ± 0.15

[a] Rats were exposed to 10 mg $CdSO_4/m^3$ for 2 h.
[b] Values represent means ± SD, n = 6 rats.

Ascorbic acid levels were also measured in PBS and $HClO_4$ nasal lavage fluid from control and Cd-exposed rats at 0 and 24 h after exposure. Ascorbic acid was expressed using either milligram lavage fluid protein, DNA, or lavage fluid volume as a denominator (Table 23). Control rats lavaged 24 h after exposure had the highest levels of ascorbic acid in both PBS and $HClO_4$ rinses. Exposure to $CdSO_4$ had no apparent effect on ascorbic acid levels.

TABLE 23. EFFECTS OF $CdSO_4$ EXPOSURE ON ASCORBIC ACID IN RAT NASAL LAVAGE FLUIDS

		ng Ascorbic Acid/mg Protein	
Group	Hours Post-Exposure[a]	PBS Rinse	PCA Rinse
Control	0	69 ± 13.1[b]	115 ± 36.3
Exposed	0	63 ± 4.2	94 ± 24.6
Control	24	158 ± 44.3	264 ± 69.4
Exposed	24	55 ± 13.9	87 ± 40.3

(continued)

TABLE 23. (Continued)

Group	Hours Post-Exposure[a]	ng Ascorbic Acid/ng DNA PBS Rinse	PCA Rinse
Control	0	5.0 ± 0.1	32 ± 16.3
Exposed	0	4.6 ± 0.2	32 ± 10.0
Control	24	15.2 ± 5.0	101 ± 33.2
Exposed	24	5.4 ± 0.9	26 ± 7.1

Group	Hours Post-Exposure[a]	µg Ascorbic Acid/2 mL Lavage Fluid PBS Rinse	PCA Rinse
Control	0	1.20 ± 0.02	3.91 ± 1.83
Exposed	0	1.28 ± 0.10	3.04 ± 0.82
Control	24	3.11 ± 0.99	6.97 ± 2.00
Exposed	24	1.27 ± 0.22	2.77 ± 0.90

[a] Rats were exposed to 10 mg $CdSO_4/m^3$ for 2 h.
[b] Values represent means ± SD, n = 6 rats.

Nose-only Cd exposure also caused an increase in the protein content of the nasal lavage fluid (Figure 20). Exposure to both 1 and 10 mg/m³ caused protein levels to increase twofold at both dose levels. When expressed as nanograms of Cd per microgram of protein, the Cd levels in the nasal lavage fluid peaked immediately after exposure and decreased rapidly with time after exposure (Figure 21). However, by eliminating the effects of the increasing protein levels by expressing the Cd levels as micrograms of Cd per milliliter lavage fluid (Figure 22), the Cd levels remained elevated after exposure with only a slight decrease after 24 h.

LUNG LAVAGE FLUID PROTEIN

Lung lavage was performed on control rats exposed to $CdSO_4$ aerosols to obtain alveolar macrophages. After removal of AMs, the lung lavage fluid was analyzed for Cd and protein to provide an index of lung injury. Lung lavage fluid protein increased with time after exposure to $CdSO_4$ (Figure 23). Exposure to 10 mg $CdSO_4/m^3$ resulted in about four times higher lavage fluid protein than exposure to 1 mg $CdSO_4/m^3$. The increased protein was most likely a result of plasma entering the lung cavity due to damage to cell membranes.

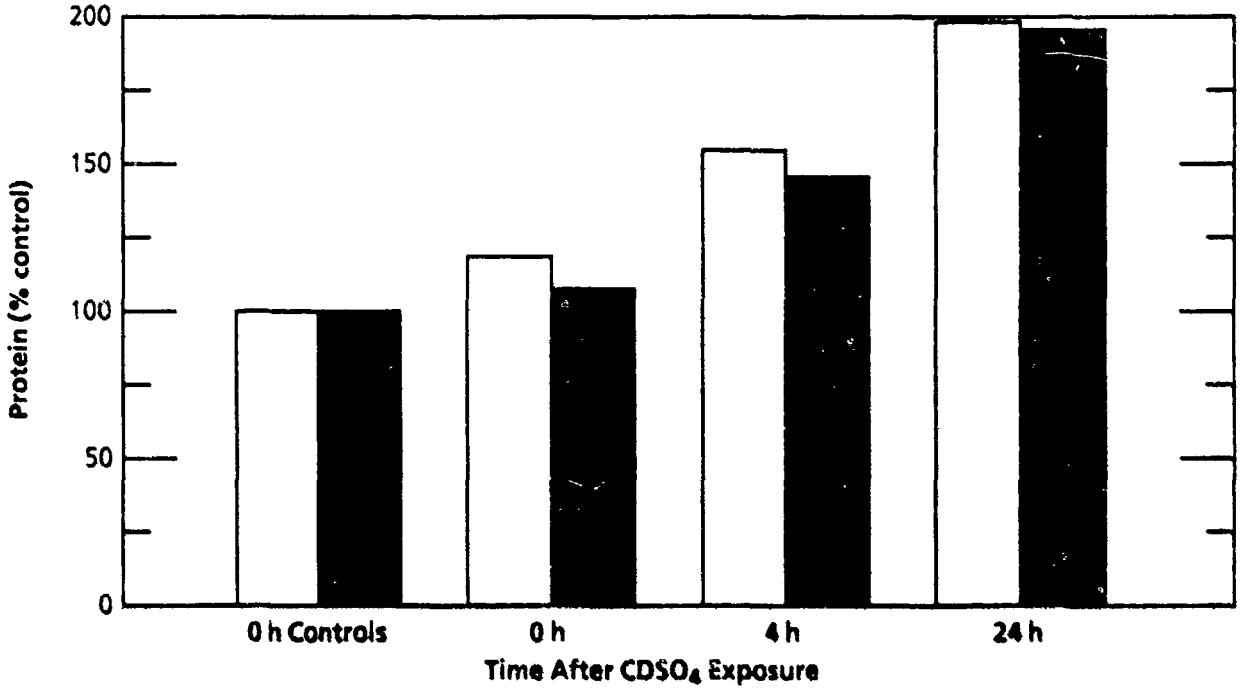

Figure 20. Protein Content of Nasal Lavage Fluid from $CdSO_4$-Exposed Rats. Six rats per group were exposed to 1 or 10 mg/m³ for 2 h. □, 1 mg/m³; ■, 10 mg/m³.

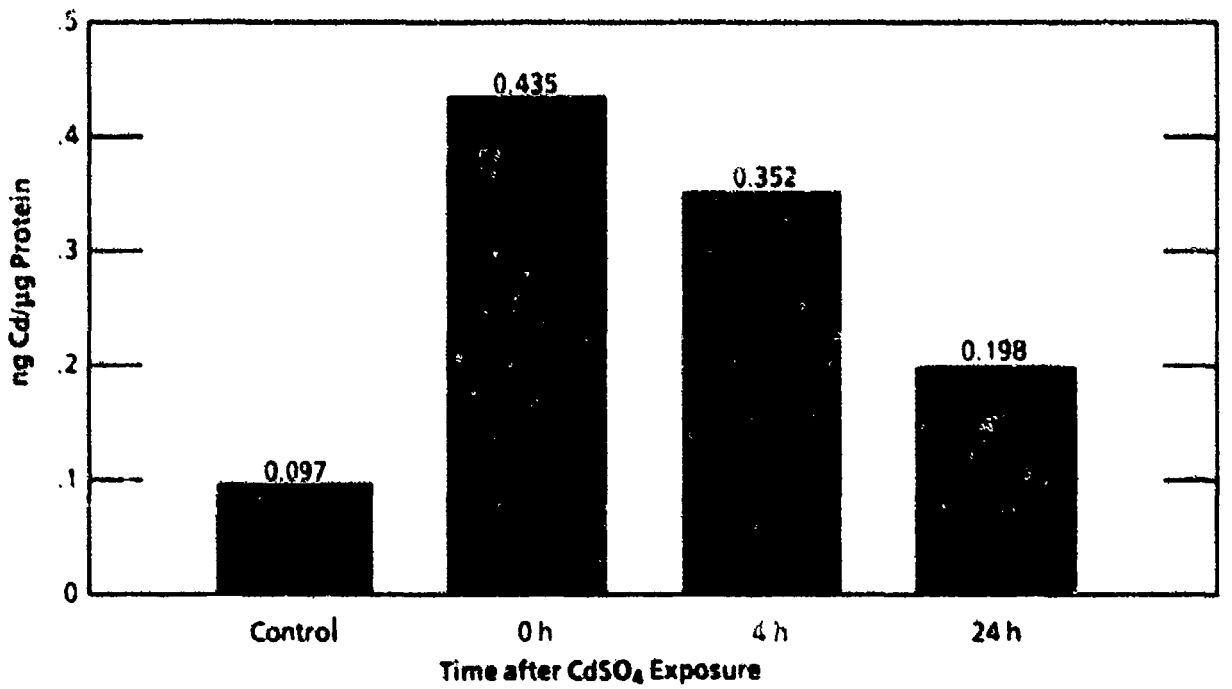

Figure 21. Cadmium Levels in Nasal Lavage Fluid of $CdSO_4$-Exposed Rats. Six rats per group were exposed to 10 mg/m³ for 2 h.

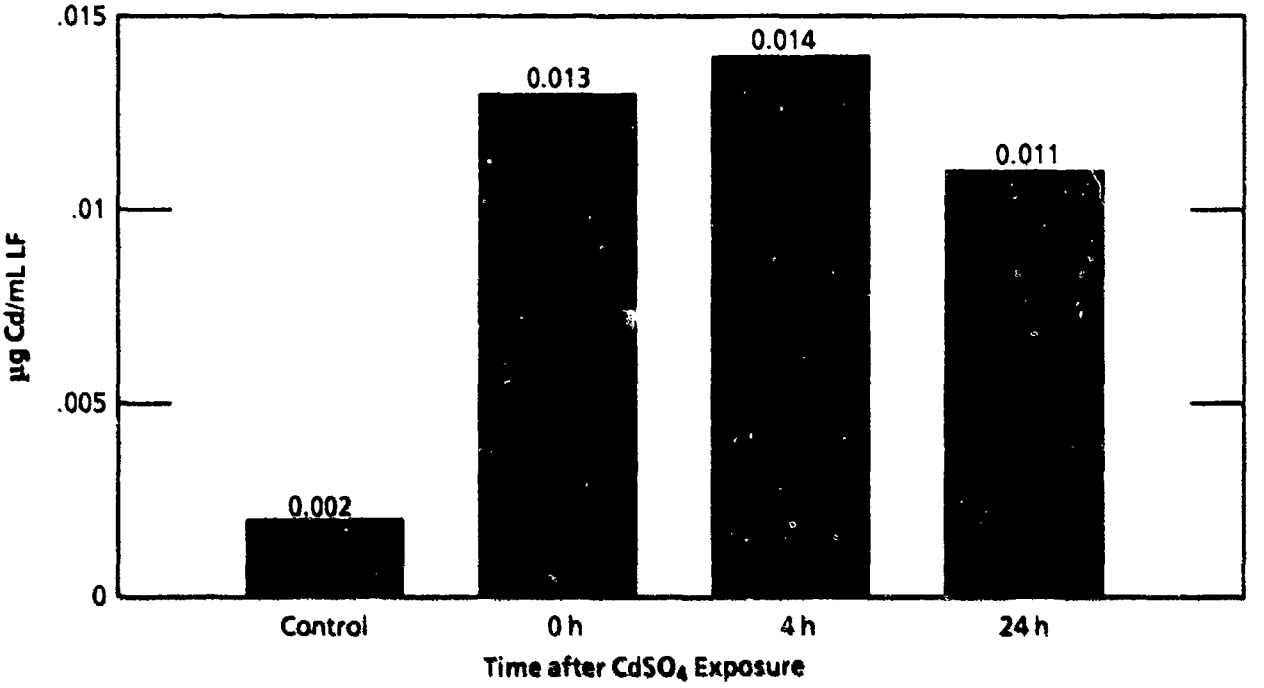

Figure 22. Cadmium Levels in Nasal Lavage Fluid of $CdSO_4$-Exposed Rats (µg/mL lavage fluid). Six rats per group were exposed to 10 mg/m^3 for 2 h.

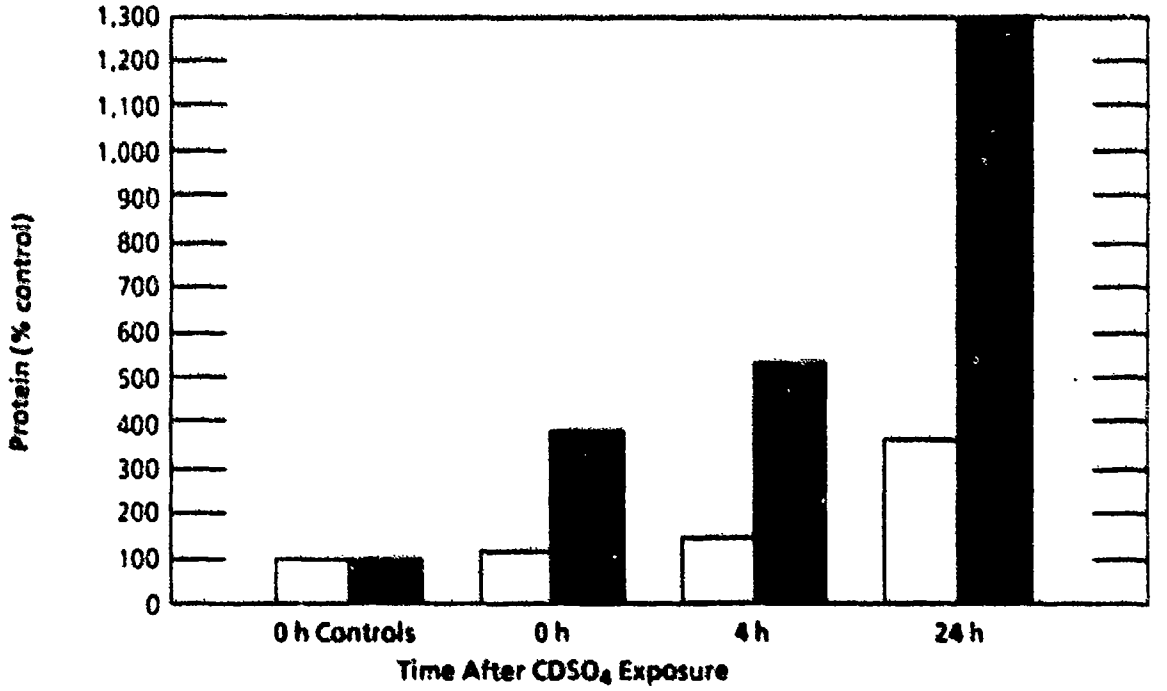

Figure 23. Protein Content of Lung Lavage Fluid from $CdSO_4$-Exposed Rats. Six rats per group were exposed for 2 h. □, 1 mg/m^3; ■, 10 mg/m^3.

ALVEOLAR MACROPHAGES

An initial experiment was performed to determine the number of AMs that could be obtained by pulmonary lavage of F-344 rats. This information was needed to determine the number of AMs/rat that were available for the different biochemical assays (Cd, nucleotides, antioxidants, protein, DNA).

Four rats were lavaged six times each with saline (35 mL/kg body weight) and the cell number determined in the lavage fluid. An average of 3.62×10^6 AMs were obtained per rat (Table 24). The lavage fluid was centrifuged and the AM pellets immediately extracted with cold 3% $HClO_4$. These AM extracts were analyzed by HPLC for nucleotides. Energy charge values for AMs were low relative to the values typically obtained for nasal epithelial cells (Table 25). Variation in EC between rats was relatively low. Based upon these cell numbers and nucleotide values, it appeared feasible to perform all biochemical analyses on an individual animal sample without having to pool AMs from different animals. Each AM sample could be divided into two parts (about 1.8×10^6 AMs): (1) for nucleotide and antioxidant analyses and (2) for Cd, protein, and DNA analyses.

TABLE 24. ADENINE NUCLEOTIDE LEVELS OF RAT MACROPHAGES[a]

Sample Number	AMs/RAT $\times 10^6$	ng Nucleotide/10^6 Alveoloar Macrophages					
		ATP	ADP	NADP	AMP	NAD	cAMP
RAT 1	3.02	3,767	5,212	2,782	3,968	17,852	907
RAT 2	3.78	6,301	5,406	3,483	4,727	18,262	641
RAT 3	3 04	6,065	5,674	3,694	4,419	18,661	812
RAT 4	4.65	4,125	3,248	2,048	2,029	12,019	574
Mean	3.62	5,564	4,885	3,002	3,786	16,698	734
SD	0.77	984	1,108	746	1,212	3,137	153

[a] Unexposed control rats.

TABLE 25. INTRASPECIES VARIATION IN ENERGY CHARGE OF ALVEOLAR MACROPHAGES[a]

Sample Number	Energy Charge	% AXP[b]		
		ATP	ADP	AMP
RAT 1	0.56	38.58	34.87	26.55
RAT 2	0.55	38.34	32.89	28.76
RAT 3	0.55	37.54	35.12	27.35
RAT 4	0.61	43.88	34.54	21.58
Mean Value	0.57	39.58	34.36	26.06
SD	0.03	2.90	1.01	3.12

[a] Unexposed control rats.
[b] A(X)P = ATP + ADP + AMP.

In Vitro Alveolar Macrophage Studies

Viability and Nucieotide Measurements

Several experiments were performed to determine the effects of *in vitro* Cd exposure on AM viability and nucleotide levels. In an initial range-finding experiment, rat AMs were exposed to 0, 0.125, 0.25, 0.5, or 1.0 mg $CdSO_4$/mL for 2 h. Cell viability (trypan blue exclusion) decreased significantly with increasing $CdSO_4$ concentration (Table 26). The total number of AMs was decreased after exposure to 1.0 mg/mL. Cellular Cd content (pg/AM) increased with increasing exposure concentration. Exposure to 1.0 mg/mL resulted in about a 500-fold increase in cellular Cd levels over AMs exposed to 0.5 mg/mL. However, a clean dose-response effect of $CdSO_4$ on EC levels was not observed.

TABLE 26. EFFECTS OF *IN VITRO* $CdSO_4$ EXPOSURE ON RAT ALVEOLAR MACROPHAGES

Exposure Concentration (mg/mL)[a]	Cellular Cd ($\mu g/10^5$ cells)	% Viable	AMs Remaining ($\times 10^5$)[b]	Energy Charge
0	0.022 ± .027[c]	98.1 ± 0.8	2.58 ± .09	.691 ± .09
0.125	0.063 ± .035	94.7 ± 2.1	2.95 ± .53	.640 ± .15
0.25	0.082 ± .010	95.3 ± 1.6	2.74 ± .65	.599 ± .21
0.50	0.296 ± .294	89.7 ± 3.2	2.13 ± .53	.621 ± .18
1.00	113.5 ± 36.5[d]	78.7 ± 12.9[d]	1.22 ± .27[d]	.673 ± .19

[a] 2-h *in vitro* $CdSO_4$ exposure.
[b] Number of intact cells remaining after the 2-h exposure.
[c] Values represent means ± SD of three cultures. AMs from four rats were pooled and then equally divided into treatment groups.
[d] Significantly different than control (0 concentration).

In subsequent experiments, human and rat AMs were exposed *in vitro* to 1.0 mg $CdSO_4$/mL for 2 h. Cadmium exposure decreased human AM viability to 11% and rat viability to 34% of control (Table 27). The EC was decreased to about the same extent in both human and rat AMs.

TABLE 27. EFFECTS OF *IN VITRO* $CdSO_4$ EXPOSURE ON HUMAN AND RAT ALVEOLAR MACROPHAGES

Subject	Exposure Concentration (mg/mL)[a]	% Viable	AMs Remaining (% Control)[b]	Energy Charge
Human	0	95	100	.800 ± .157[c]
	1	11	107	.603 ± .145
Rat	0	99	100	.817 ± .115
	1	34	88	.686 ± .096

[a] *In vitro* exposure for 2 h
[b] The numbers of AMs remaining in human and rat control cultures were 4.1×10^5 and 3.6×10^5, respectively.
[c] Values represent means ± SD of three cultures.

DNA Damage

An initial range-finding study was performed to determine the concentrations of $CdSO_4$ required to cause detectable DNA damage to AMs *in vitro*. Macrophages were isolated from untreated animals and exposed to $CdSO_4$ *in vitro*. Macrophages from four animals were isolated, centrifuged, resuspended in PBS, and counted. Each sample contained approximately three million cells. The AMs were exposed to 0, 0.05, 0.5, or 5 mg $CdSO_4$/mL in 2 mL of PBS for 2 h. The elution of the DNA from these cells is shown in Figure 24. Over a 15-h elution period, 55% of the control DNA passed through the filter. Also, DNA from cells exposed to 0.05 mg/mL showed a similar pattern, with 60% of the DNA passing through the filter. For cells exposed to 0.5 and 5.0 mg Cd/mL, 83% and 88% of the DNA eluted, respectively. From these experiments, it seemed possible to observe a dose response with this assay in AMs exposed *in vitro* to Cd.

The dose response in a narrower and lower range was examined to correlate with concentrations being used in the nucleotide assays. Rat AMs were exposed to 0.25, 0.5, and 1 mg Cd/mL (Figure 25). The DNA from control cells eluted at an abnormally rapid initial rate for reasons unknown. However, a typical dose response can still be seen in treated groups.

DNA damage to human AMs following $CdSO_4$ exposure was also evaluated by the alkaline elution assay. Two sample aliquots were exposed to 1 mg Cd/mL for 2 h and two aliquots were used as controls. The results were very similar within duplicates of each group (Figure 26). The two control samples eluted to 63.9% and 64.4%, respectively (typical values for control DNA). The Cd-treated samples eluted to 53.2% and 51.5%, respectively. Unfortunately, attempts to secure an additional sample of human macrophages to repeat these studies were unsuccessful.

In Vivo Alveolar Macrophage Studies

In initial studies, AMs were obtained by lung lavage from F-344 rats at 0, 4, or 24 h after nose-only exposure to either 1 or 10 mg $CdSO_4/m^3$ (MMAD = 0.1 μm) for 2 h. The number of AMs obtained by lung lavage was markedly decreased by Cd exposure (Figure 27). Exposure to 1 mg $CdSO_4/m^3$ produced a 40% decrease in the number of AMs obtained by lung lavage at 0 and 4 h after exposure. The yield of AMs after exposure of rats to 10 mg/m^3 was decreased to about 10% of control immediately after exposure, and had recovered slightly by 24 h after exposure.

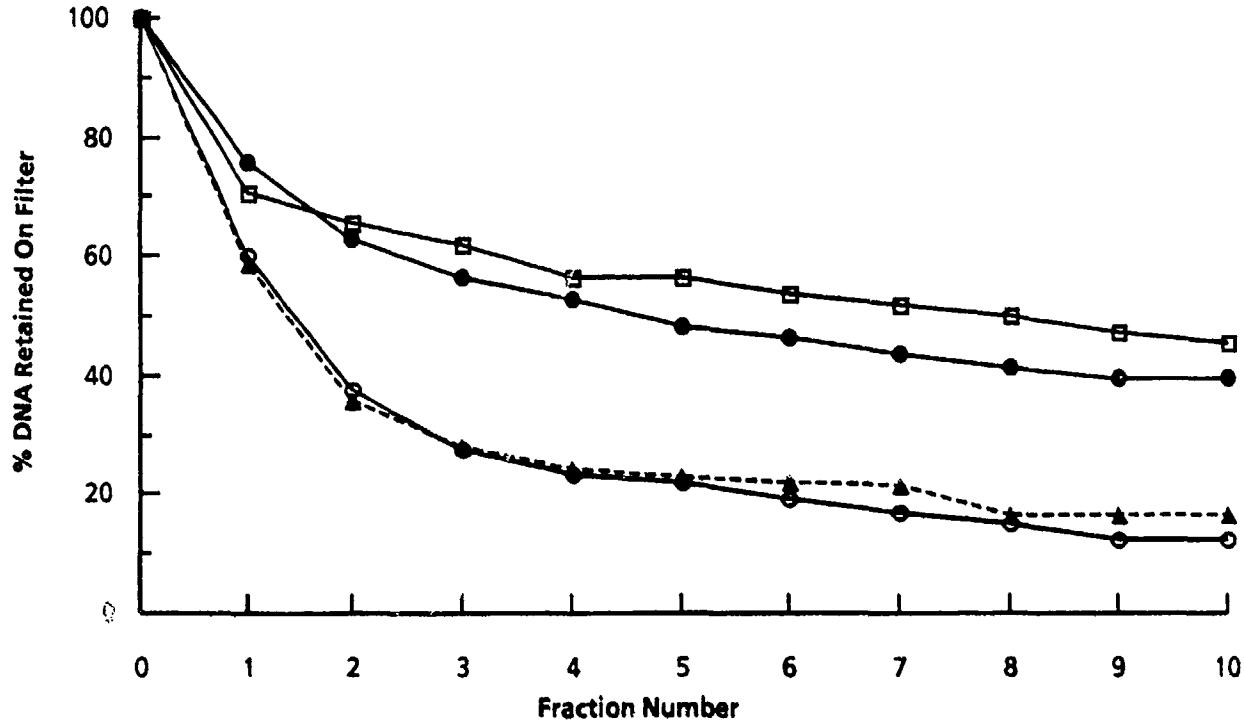

Figure 24. Alkaline Elution Assay of DNA from Rat Pulmonary Macrophages Exposed for 2 h *In Vitro* to $CdSO_4$. Experiment 1. □, 0 mg/mL; ●, 0.05 mg/mL; ▲, 0.5 mg/mL; ○, 5 mg/mL.

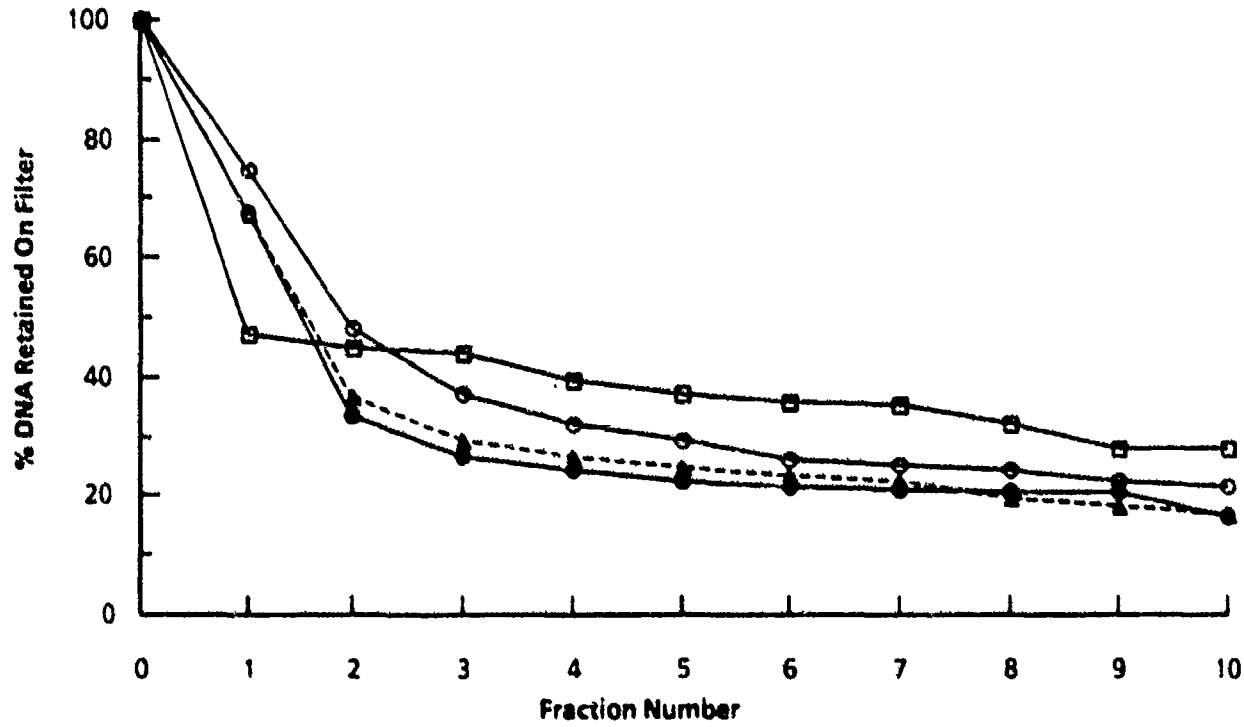

Figure 25. Alkaline Elution Assay of DNA from Rat Pulmonary Macrophages Exposed for 2 h *In Vitro* to $CdSO_4$. Experiment 2. □, 0 mg/mL; ○, 0.25 mg/mL; ▲, 0.5 mg/mL; ●, 1 mg/mL.

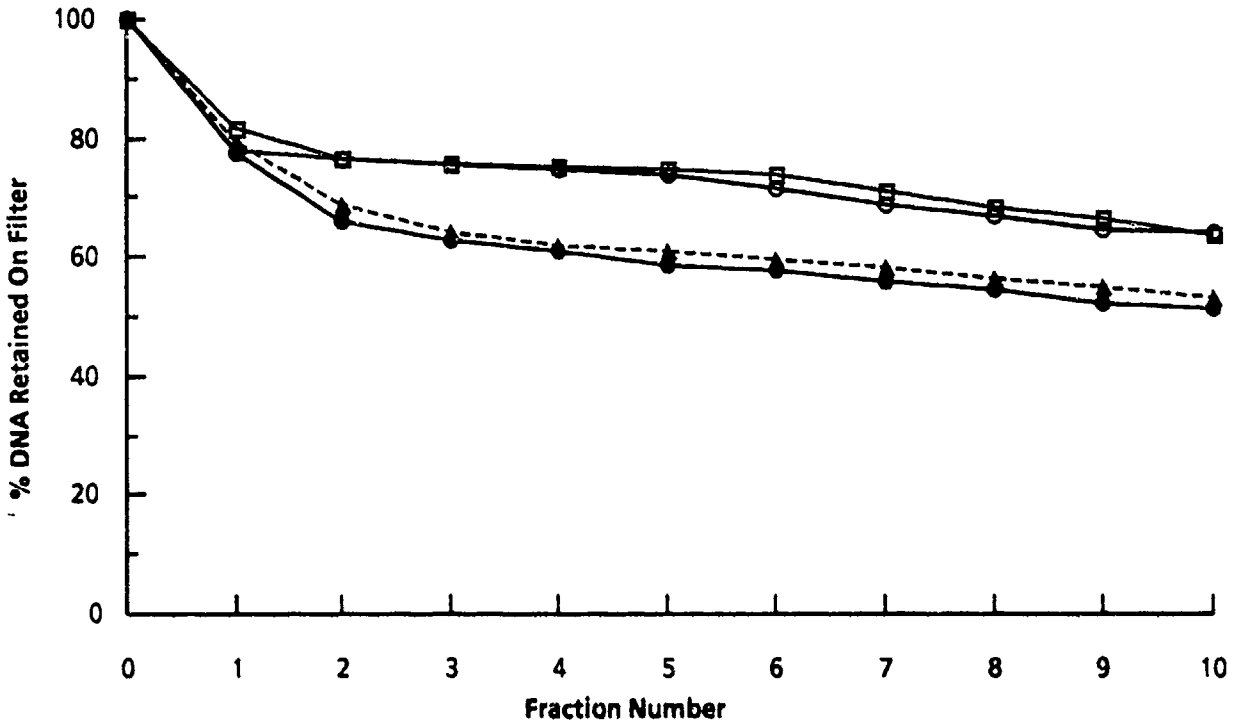

Figure 26. Alkaline Elution Assay of DNA from Human Pulmonary Macrophages Exposed for 2 h *In Vitro* to $CdSO_4$. □, 0 mg/mL; ○, 0 mg/mL; ▲, 1 mg/mL; ●, 1 mg/mL.

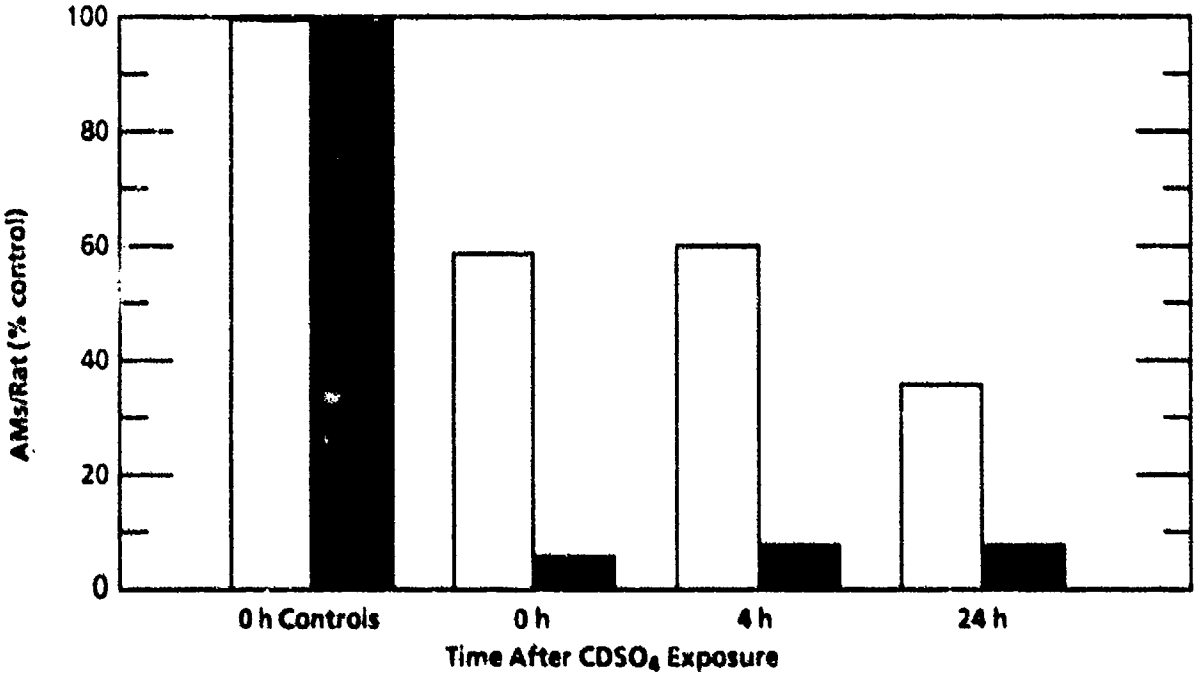

Figure 27. Number of Alveolar Macrophages Obtained from $CdSO_4$-Exposed Rats. Six rats per group were exposed to $CdSO_4$ for 2 h. □, 1 mg/m^3; ■, 10 mg/m^3.

Energy Charge and Nucleotide Levels

The EC of AMs from rats exposed to 1 mg/m^3 decreased to about 60% of control levels by 4 h after exposure (Figure 28), whereas the maximum decrease in EC for AMs from rats exposed to 10 mg/m^3 was only 15% and occurred immediately after exposure. Exposure to 1 mg/m^3 appeared to have a greater effect on AM EC than exposure to 10 mg/m^3. By 24 h after exposure, the EC levels had returned to control for AMs from rats exposed to either 1 or 10 mg $CdSO_4$/m^3.

The effects of Cd on the individual adenine nucleotides used to calculate AM ECs are shown in Figure 29. For both Cd exposure concentrations, the ATP levels decreased and AMP levels increased at 0 and 4 h after exposure. ADP levels were increased with time after exposure 1 mg Cd/m^3, but not for the 10 mg/m^3 exposure. The ATP:ADP:AMP ratios had returned to near control levels by 24 h after exposure.

Antioxidant Determinations

Antioxidant levels were measured for AMs from rats exposed to 1 mg Cd/m^3 for use as a potential endpoint for extrapolation (Figure 30). Ascorbic acid and uric acid levels were not changed significantly by Cd exposure. However, reduced glutathione levels were increased with time after exposure to almost tenfold higher by 24 h after exposure.

Cadmium Analysis

Alveolar macrophages from rats exposed to 10 mg Cd/m^3 were analyzed for Cd content (Figure 31). A 100-fold increase in Cd content was detected in intact AMs immediately after exposure. This level decreased from about 160 ng/cell to about 50 ng/cell by 24 h after exposure.

DNA Damage In Vivo

DNA damage was chosen as another endpoint for these extrapolation studies, because such damage can lead to long-term chronic effects. DNA damage was analyzed in AMs exposed *in vivo* to $CdSO_4$. The AMs were rinsed from the animals, washed once in PBS, and placed on dry ice for temporary storage until analysis. Later, the alkaline elution analysis revealed that the process of rapidly freezing the cells had broken the DNA molecules to small sizes which rapidly eluted through the filters (Figure 32). No differences were seen between the exposed and unexposed macrophages in these experiments. These results indicated future caution be taken not to freeze the cells prior to analysis for DNA damage.

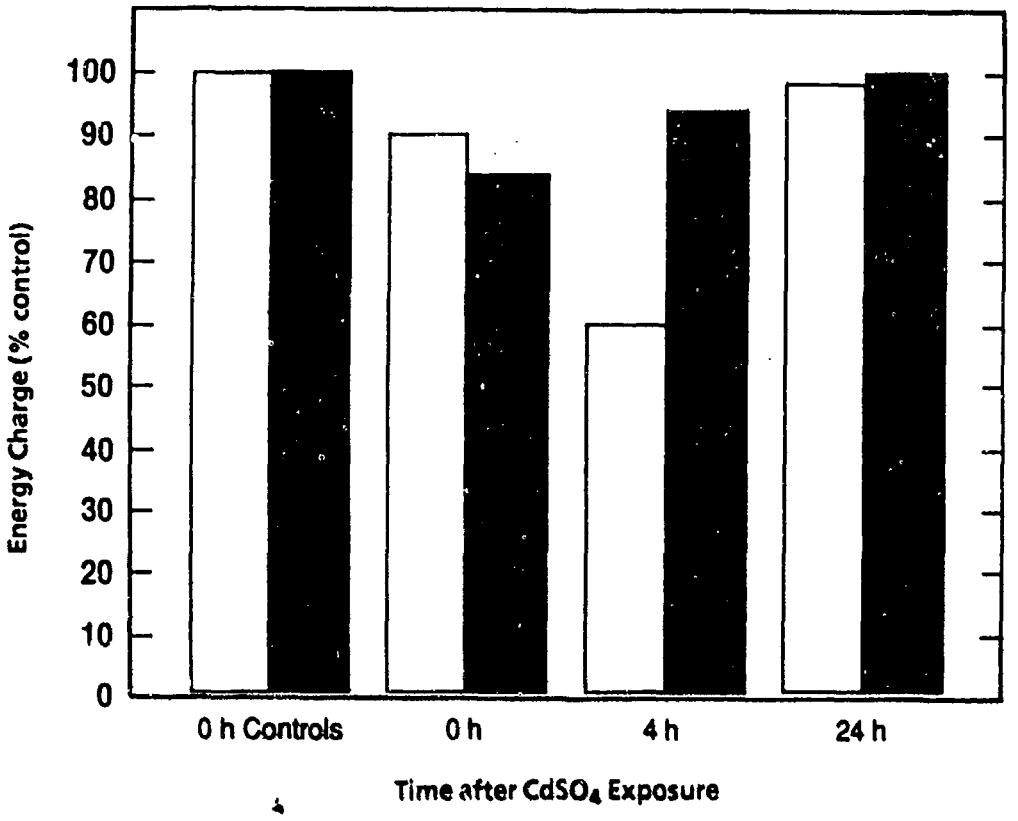

Figure 28. Energy Charge of Alveolar Macrophages from $CdSO_4$-Exposed Rats. Six rats per group were exposed to $CdSO_4$ for 2 h. □, 1 mg/m³; ■, 10 mg/m³.

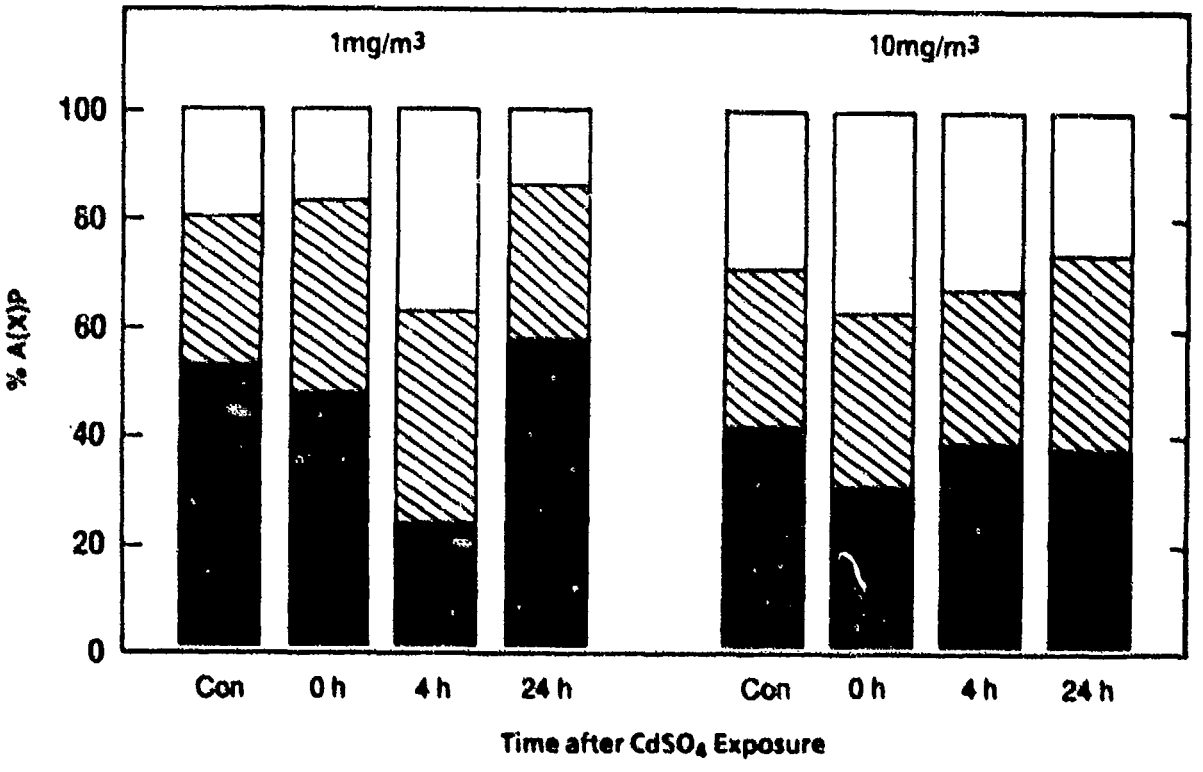

Figure 29. Adenine Nucleotides of Alveolar Macrophages from $CdSO_4$-Exposed Rats. Six rats per group were exposed to $CdSO_4$ for 2 h. □, AMP; ▨, ADP; ■, ATP.

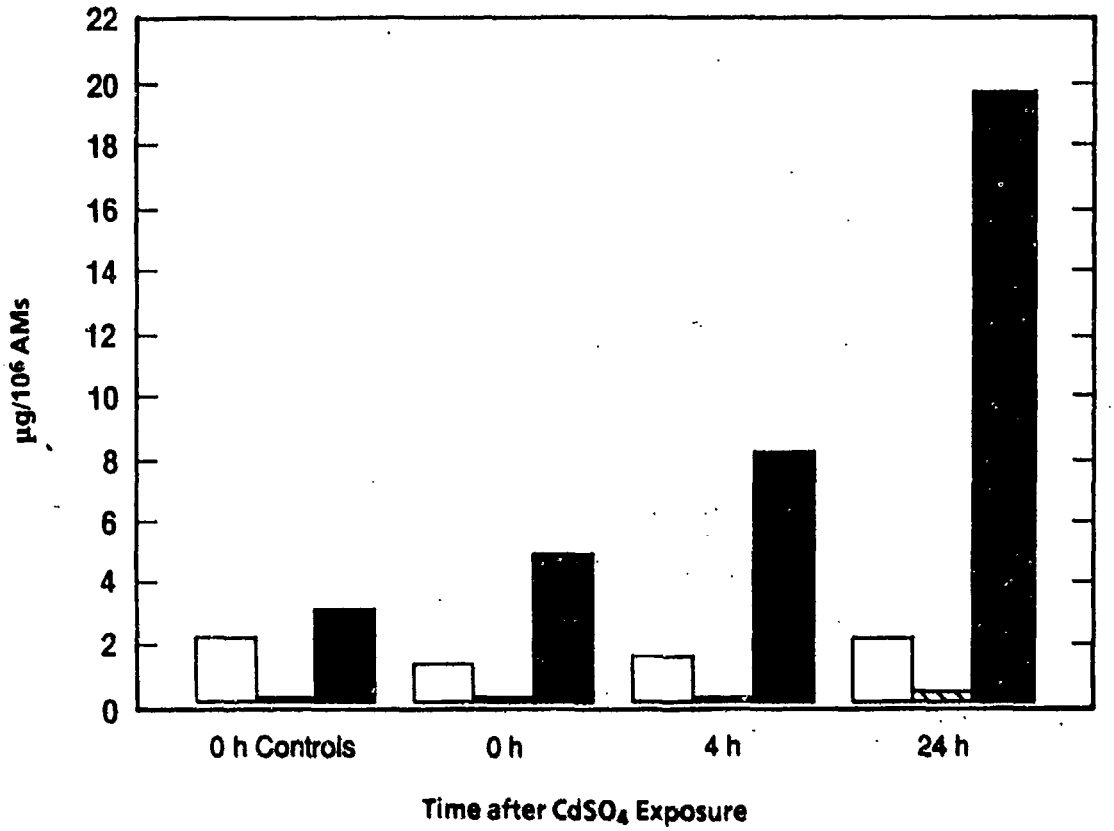

Figure 30. Antioxidant Content of Alveolar Macrophages from $CdSO_4$-Exposed Rats. Six rats per group were exposed to 1 mg $CdSO_4/m^3$ for 2 h. □, Ascorbic acid; ▨, Uric acid; ■, glutathione.

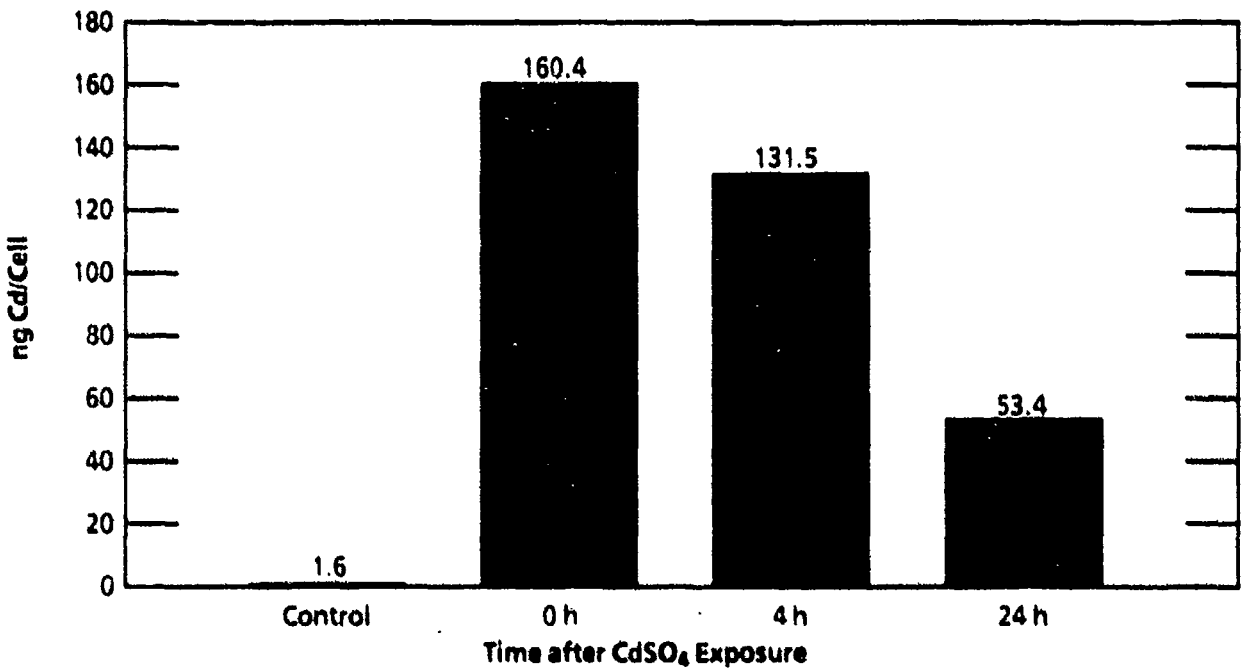

Figure 31. Cadmium Levels in Alveolar Macrophages from $CdSO_4$-Exposed Rats. Six rats per group were exposed to 10 mg $CdSO_4/m^3$ for 2 h.

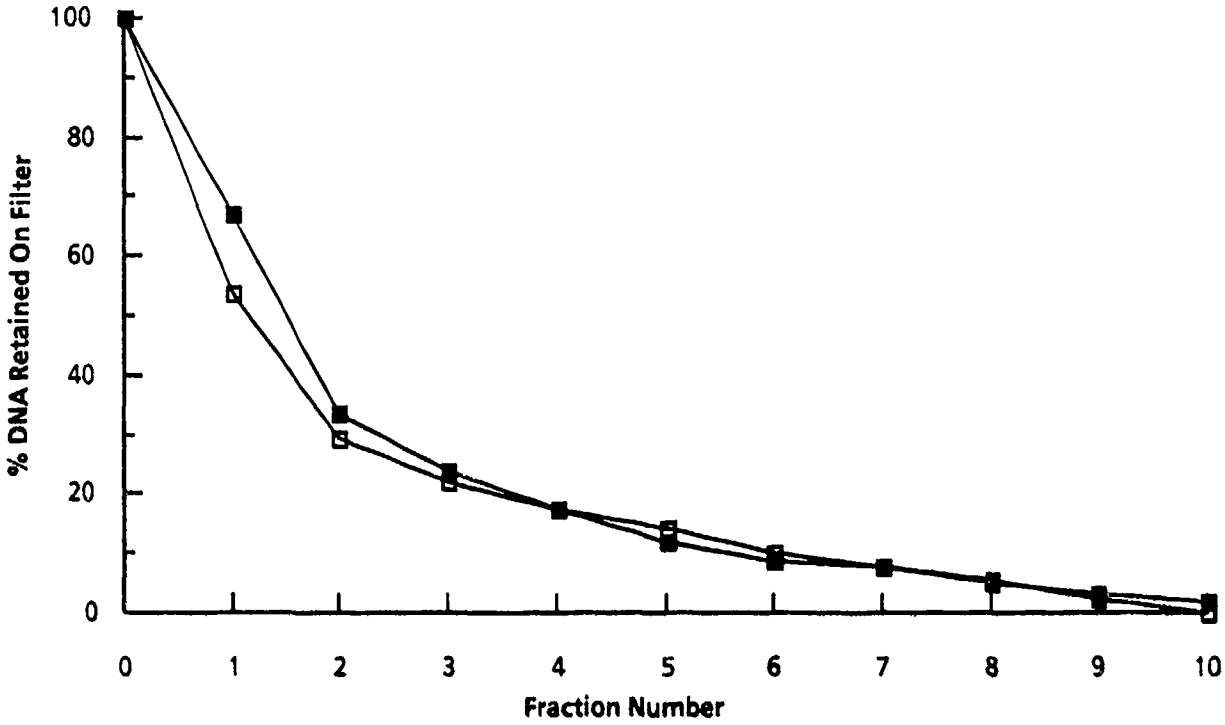

Figure 32. Alkaline Elution Assay of DNA from Rat Pulmonary Macrophages Exposed *In Vivo* to $CdSO_4$. Experiment 1. □, 0 mg/m³; ■, 1 mg/m³.

In a second *in vivo* exposure experiment, animals were exposed to 1 mg/m³ $CdSO_4$ for 2 h, or to normal air. The animals were sacrificed and the AMs isolated by lavage. The DNA from control AMs eluted unusually rapidly, leaving only 32 to 35% on the filter after 10 fractions (Figure 33). The DNA from $CdSO_4$ exposed AMs eluted only slightly faster, with 30% remaining on the filter after elution.

A summary of the *in vitro* and *in vivo* AM alkaline elution data is shown in (Table 28). To evaluate the relative amount of DNA damage, the amount of DNA from control AMs remaining on the filter was subtracted from the DNA remaining on the filter from treated AMs for each fraction. Since treated DNA was broken into smaller pieces by Cd, less remained on the filter compared to control cell DNA, and therefore, nearly all of the differences were negative. We also wanted to simplify the values for all the fractions to a single number for interspecies comparative purposes. To eliminate first fraction variability, only the values for fractions two to ten were averaged. Columns A, B, and C in Table 28 represent relative Cd-induced DNA damage data from (A) rat AMS exposed *in vivo*, (B) rat AMs exposed *in vitro*, or (C) human AMs exposed *in vitro*. The fifth column represents the comparison of *in vivo* versus *in vitro* exposure methods. These data indicate that the DNA damage from AMs exposed *in vivo* to 1 mg $CdSO_4$/m³ was only about one-half that seen in rat AMs exposed to 1 mg $CdSO_4$ *in vitro*. Importantly, the last column shows that the amount of DNA damage to human and rat AMs by Cd is nearly the same following exposure to similar

concentrations. Normalization of this data using nanograms of Cd per cell data has led to cell sensitivity extrapolation values for correlation 2 on Figure 1. Exposure of human AMs to 1 mg/mL resulted in about a 12.44% increase in the elution of DNA. It is of great importance that this value is very similar to that seen for rat AMs exposed to the same concentration. The actual Cd uptake values for the two types of cells were also very similar (i.e., 0.8 ng/cell for rat AMs vs. 0.6 ng/cell for human). Therefore, the data from these studies indicates that the value for correlation 2 in Figure 1 is 1.28, with cell sensitivity measured in terms of Cd's ability to cause single-stranded breaks in AMs based on equal intracellular doses (Figure 34).

To arrive at this extrapolation value the ratio of mean % decrease of human *in vitro*/rat *in vitro* (0.96) was divided by the Cd per cell ratios of human/rat (0.6/0.8 = .75).

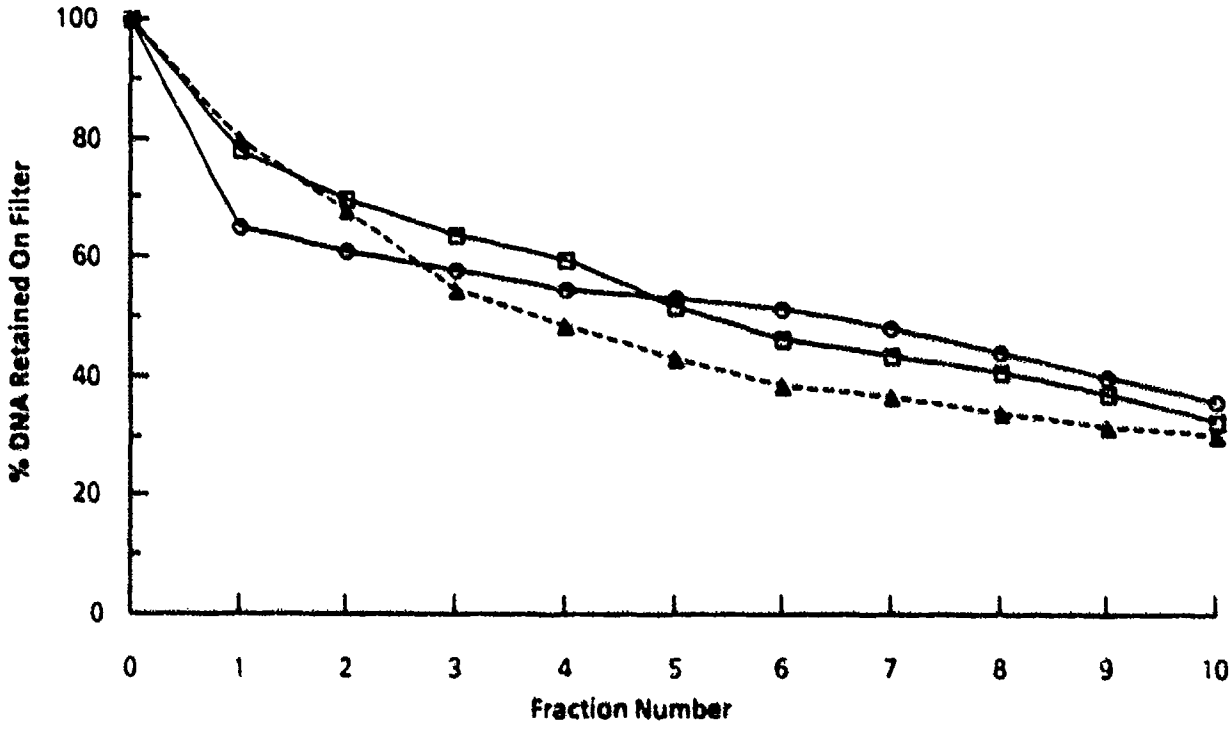

Figure 33. Alkaline Elution Assay of DNA from Rat Pulmonary Macrophages Exposed to *In Vivo* $CdSO_4$. Experiment 2. □, 0 mg/m³; ○, 0 mg/m³; ▲, 1 mg/m³.

TABLE 28. SUMMARY TABLE OF ALKALINE ELUTION DATA FROM RAT AND HUMAN ALVEOLAR MACROPHAGES

Fraction Number	Percent DNA Retained on Filter (Experimental Value[a] - Control Value)				
	Rat *In Vivo* Exposed (A)	Rat *In Vitro* Exposed (B)	Human *In Vitro* Exposed (C)	Rat *In Vivo*/Rat *In Vitro* (A)/(B)	Human *In Vitro*/ Rat *In Vitro* (C)/(B)
0	0.00	0.00	0.00	0.00	0.00
1	8.15	20.05	-1.58	0.41	0.079
2	2.38	-11.53	-9.03	-0.21	0.783
3	-6.53	-17.41	-12.14	0.38	0.697
4	-8.52	-15.03	-13.54	0.57	0.901
5	-9.51	-14.61	-14.14	0.65	0.968
6	-10.06	-14.36	-13.96	0.70	0.972
7	-9.31	-14.16	-12.94	0.66	0.914
8	-8.32	-11.22	-12.49	0.74	1.11
9	-6.45	-7.12	-11.94	0.91	1.68
10	-3.89	-11.35	-11.76	0.34	1.04
Mean Value[b]	-6.69	-12.98	-12.44	0.53	0.96

[a] Exposed to 1 mg/m^3 *in vivo* or 1 mg/mL *in vitro*.
[b] Average of fractions 2-10.

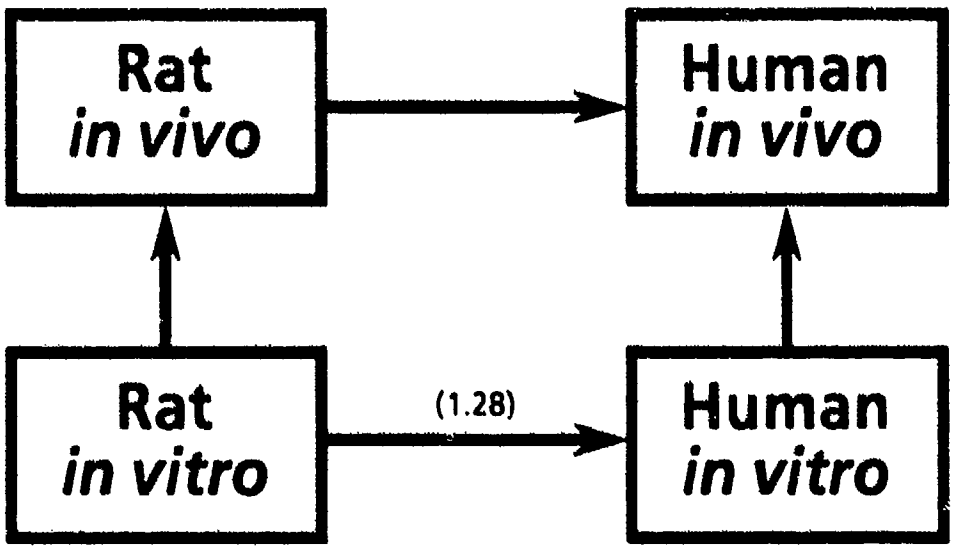

Figure 34. Tissue sensitivity extrapolation of cadmium-induced DNA damage data from rat pulmonary macrophages to human macrophages. The response is normalized for cadmium/cell.

DISCUSSION

The objective of this project was to obtain the tissue sensitivity data necessary to extrapolate quantitatively the toxic effects of inhaled particles from animals to humans. A considerable amount of effort was devoted to methods development because of the unique problem and the requirement for highly sensitive measurements. Methods were required that were directly applicable to both *in vivo* and *in vitro* models, could be used for different species, and could be used to measure and correlate directly the absorbed tissue dose of a toxicant with a toxicity endpoint in the same small tissue sample. Analytical methods that satisfied these requirements were obtained by modifying reported methods and developing new methods specifically for this project. The following paragraphs summarize the procedure modifications.

CELL CULTURE

Because the culture of NTE cells to a large extent was developed before this project began, efforts were focused on improving existing culture systems to (1) grow the NTE cells from rat and humans in common media, (2) determine the approximate dose of Cd that causes toxicity to epithelial cells, (3) improve methods for harvesting human and animal nasal epithelial cells, and (4) grow the NTE cells without substrate requirements that interfered with biochemical analyses.

Methods developed previously for isolation and culture of cells from the upper airways of animals and from human respiratory tissue (Steele and Arnold, 1985) were modified slightly and utilized for both rat and human NTE cells. Differences in cell isolation and culture methods for rat and human cells were eliminated as much as possible to allow a more accurate determination of species sensitivity and to facilitate the extrapolation of toxic effects between the two species. Methods were modified so that both rat and human NTE cells could be isolated by an explant/outgrowth method, cultured in medium containing the same nutrients and growth factors, and grown without a collagen film substrate.

HPLC ANALYSIS OF NUCLEOTIDES

Previously, nucleotides have been analyzed by a variety of techniques. An HPLC method was chosen to analyze the adenine nucleotide pool, primarily because of the high specificity and sensitivity of the technique (detection limit at t = 254 nm = 1 ng/injection). Several HPLC methods for analyzing nucleotides using anion exchange columns and gradient elution have been published. Because retention times for nucleotides are very long when using anion exchange, reverse-phase HPLC methods were investigated. Various reverse-phase HPLC assays were tried and modified before arriving at an acceptable method.

A considerable amount of effort was devoted to the development of the HPLC methodology required to analyze low levels of nucleotides in very small samples. Because adenine nucleotides are extremely labile, all efforts were made to prevent enzymatic and nonenzymatic degradation, and interconversions of these compounds during sample preparation. Not only was high sensitivity required, but also a relatively rapid run time.

DNA DAMAGE ENDPOINT

The alkaline elution method for quantitating DNA damage was adapted so that a reliable microfluorometric procedure could be used to quantitate the DNA instead of prelabeling with DNA precursors. This procedure is far more economical than procedures involving radioactive compounds and appears equally as sensitive.

***IN VITRO* EXPOSURE METHODS**

Nasal turbinate epithelial cells were exposed *in vitro* by dissolving the $CdSO_4$ in a suitable medium and adding this solution to the cells in culture dishes. A number of factors were considered in selecting a suitable exposure medium. In addition to meeting the osmolarity and nutrient requirements of the cells, the exposure medium had to be a solution in which $CdSO_4$ was freely soluble and that had a strong buffering system to prevent pH changes that occurred at high $CdSO_4$ concentrations. Proteins such as BSA and FBS which readily bind Cd^{2+}, thereby decreasing the amount available to the cells, were not included in the NTE cell exposure medium. Also considered was the presence of the divalent cations Ca^{2+} and Mg^{2+}, which can compete with Cd^{2+} for receptors on cell membranes, and may also affect the solubility of $CdSO_4$.

Because Ham's F12 was the basic component of the growth medium used for both human and rat NTE cells, this medium was selected as the exposure medium for NTE cells *in vitro*. Although Ham's F12 contains Ca^{2+} and Mg^{2+}, the levels were not high enough to compete with the considerably higher concentrations of Cd^{2+}. At the highest $CdSO_4$ concentration used for NTE cell exposures (1.0 mg/mL), precipitation and pH changes were not a problem.

After *in vitro* Cd exposure, cell cultures were rinsed three times to remove the Cd treatment medium and excess extracellular Cd before extracting cellular nucleotides. In addition to removing excess Cd, this rinsing procedure may also remove cells that were damaged by Cd and detached from the culture dishes. Because these injured, detached cells would be expected to have lower EC values than the attached cells, rinsing them off the dish may have decreased the sensitivity of detecting an EC decrease in the entire culture.

It was determined that rinsing and removing detached cells does not selectively remove Cd-injured cells with low EC levels. A decrease in EC caused by Cd was still detected in the cells that

remained attached to the dishes after rinsing. Rinsing appeared to enhance the effects of Cd on EC. These unexpected results were due to increases in ATP and AMP levels (in control cultures only) caused by rinsing cells. The removal of detached cells by rinsing did not appear to be an important factor in evaluating Cd effects on the EC of NTE cells.

TISSUE SENSITIVITY DETERMINATIONS

Nasal Epithelium – *In Vitro* Exposures

Differences in rat and human cell sensitivities to $CdSO_4$ were evaluated *in vitro* using EC and changes in levels of nucleotides per nanogram DNA as toxicity endpoints. Significant differences were observed in the Cd content of exposed human and rat NTE cells. Human cell extracts contained eight to ten times more Cd than rat cell extracts after a 2-h exposure to 1 mg/mL Cd. Because intracelluiar and membrane-bound Cd were not differentiated, it was not possible to determine if these differences in Cd content were due to differences in cellular uptake or membrane binding. Although human NTE cells were found to have about 1.5 times higher levels of protein-bound SH than rat NTE cells (Table 1), it is unlikely that this relatively small difference in SH levels could account for the large fivefold difference in Cd levels between the two cell types.

Although human cell extracts contained higher Cd levels than rat cell extracts after exposure for only 30 min, the EC was not significantly lower for human cells until after 240 min of exposure. These data suggest that differences in total cellular Cd levels may have been due primarily to higher membrane-bound levels in human cells. Membrane-bound Cd may have much less effect on cellular EC than intracellular Cd. Intracellular Cd could directly affect mitochondrial respiration, resulting in a decreased EC. Energy charge levels were similar for both cell types for most of the exposure, suggesting that intracellular Cd levels were similar, while membrane-bound Cd levels were possibly much higher for human cells.

When cells were exposed for 2 h to a range of $CdSO_4$ concentrations, Cd levels were significantly different at the highest concentration (4.8 mM) for rat and human cell extracts, but similar at lower concentrations. A similar trend was observed in EC measurements, which also appeared to differ between rat and human cells only at the highest Cd concentration. Exposure to 4.8 mM $CdSO_4$ for 2 h produced significantly higher Cd levels and also a significantly lower EC in human cells than in rat cells.

Changes in nucleotide levels were dependent on Cd concentration and duration of exposure In general, ATP, ADP, and NAD levels decreased while AMP levels increased in response to an increase in Cd concentration or exposure time. These data suggest that ATP was still being utilized by the Cd-exposed cells, but that regeneration of ATP from ADP and AMP were inhibited. Although loss of cellular ATP can occur in response to a number of causes, these results are consistent with

reports demonstrating inhibition of cellular respiration caused by Cd exposure (Kisling et al., 1987; Diamond and Kench, 1974; Mustafa and Cross, 1971).

In both cell types, ATP, ADP, and AMP levels were increased initially after adding $CdSO_4$ (Tables 10,11), and decreased with time of exposure. AMP levels remained elevated above control levels. This initial increase in adenylate concentration in response to Cd exposure has been reported previously (Muller and Ohnesorge, 1984) and was attributed to a nonspecific enhancement of glucose utilization.

The effects of Cd exposure time as measured by nucleotide levels/DNA demonstrated a 74% decrease in ATP/DNA in human cells and only a 44% decrease in rat cell ATP/DNA after exposure for 120 min to 4.8 mM $CdSO_4$. These data agree with EC data indicating that human NTE cells may indeed be more sensitive to the cytotoxic effects of Cd than rat NTE cells.

Human NTE cells were found to absorb much higher levels of Cd than rat NTE cells; however, based on cellular EC, the tissue sensitivities of human and rat NTE cells to *in vitro* Cd exposure were similar except after long exposure times and high Cd concentrations.

The alkaline elution technique was found to be a good indicator of DNA damage in rat NTE cells. Several experiments indicated that a dose response could be measured using DNA from cells exposed to various concentrations of $CdSO_4$. The results also indicated that the 2-h exposure regimen (1 mg/mL concentration) used for the other studies induced measurable amounts of DNA damage.

Nasal Epithelium – *In Vivo* Exposures

Rat nasal epithelial cell sensitivity was also evaluated after *in vivo* $CdSO_4$ exposure using EC and nucleotide levels as toxicity endpoints. Only slight decreases could be detected in EC of rat nasal epithelium after exposure to a maximum dose of 10 mg $CdSO_4/m^3$ for 2 h. Although this effect of Cd on EC between control and exposed values was small, the difference appeared to be significant and reproducible.

The nasal epithelium *in vivo* may be relatively resistant to injury from inhaled toxicants because of the protection provided by the layer of mucus. During nose-only $CdSO_4$ exposure, about 75% of the Cd deposited in the nose appeared to be trapped in the mucus layer (Table 21). Even though $CdSO_4$ is very water-soluble, the amount bound, or taken up, by the nasal epithelium, was very small and apparently limited to a great extent by the mucus layer. This protective mechanism may be the main obstacle to measuring toxic effects in the nasal epithelium at reasonable $CdSO_4$ concentrations.

Another possible explanation for the failure to detect significant effects of $CdSO_4$ on EC in the rat nasal epithelium *in vivo* is that the high concentration of $CdSO_4$ used in the nose-only exposures caused extensive damage to the thin epithelial layer, and nucleotide measurements had to be made (due to methodological limitations of sampling) on the epithelium, plus several layers of underlying (and possibly undamaged) tissue. Alternatively, the levels of $CdSO_4$ penetrating the mucus layer and reacting with the nasal epithelium may have been too low to cause measurable toxic effects.

Histological examination of rat nasal tissues demonstrated that exposure to 10 mg $CdSO_4/m^3$ caused histological damage to the olfactory epithelium with little detectable damage to the respiratory epithelium of the nasal cavity. The lack of damage to the respiratory epithelium supported the biochemical data from similar exposures which demonstrated only slight decreases in the EC of rat nasal respiratory epithelium. This selective effect on the olfactory tissue may have been due to a lack of mucus-producing cells (no protective layer of mucus), possibly the presence of fewer cilia resulting in slower clearance of Cd, or a differential cell sensitivity between olfactory and respiratory cells.

Alveolar Macrophages – *In Vitro* Exposures

Because Cd exposure caused little toxicity for rat nasal respiratory epithelial cells *in vivo*, experiments using AMs were initiated. Alveolar macrophages may be more susceptible to Cd toxicity than NTE cells, since NTE cells appear to be protected by the layer of mucus in the nose, whereas a similar mucus layer is not present in the alveoli.

Tissue sensitivity to $CdSO_4$ was measured in rat AMs exposed *in vitro* by measuring cell viability, number of cells remaining, and EC. Although initial antioxidant measurements in AMs appeared very promising, there was not sufficient time to explore this endpoint thoroughly.

In vitro exposure of rat AMs to 10 mg $CdSO_4$/mL significantly decreased cell viability and number of AMs remaining after 2 h. Energy charge was also decreased, although not to the extent that cell viability was affected.

Rat AMs exposed to $CdSO_4$ *in vitro* sustained concentration-dependent increases in DNA damage as measured by alkaline elution analysis. The DNA from Cd-exposed cells eluted about 12.98% faster than DNA from control cells. In these experiments, the lowest concentration where damage was observed was at 0.25 mg $CdSO_4$/mL. Because of the large difference in intercellular dose between AMs exposed *in vivo* to 1 mg/m^3 (0.0035 pg/cell) and AMs exposed *in vitro* to 1 mg/mL (0.8 ng/cell), no attempt was made to estimate a value for correlation 1 (Figure 1).

A limited number of human AMs were obtained from one non-smoking patient for use in these studies. Because of the low number of AMs provided, only one $CdSO_4$ exposure concentration

was used. Based upon these limited data, human AMs appeared slightly more susceptible than rat AMs to the effects of Cd on cellular viability. Exposure to $CdSO_4$ caused an 88% loss of human AM viability and a 65% decrease in rat AM viability. Although EC was decreased slightly more in human AMs (25%) than in rat AMs (16%), this difference was not significant.

Human DNA from AMs also showed dose-dependent increases in elution rate following exposure to $CdSO_4$. These increased elution rates were normalized for cellular dose of Cd and compared to rat AM data, also normalized for cellular Cd. The resultant value of 1.28 could be used for correlation 2 in Figure 1. This is the first time a number for any biological response endpoint has been determined for extrapolating animal cell responses to human cells, based on equal intracellular doses. It is unfortunate that these studies could not have been repeated to confirm the findings, since they are so very important to progress in the field of animal to human extrapolation.

Alveolar Macrophages – *In Vivo* Exposures

In vivo $CdSO_4$ exposure decreased the number of AMs obtained by pulmonary lavage. Exposure to 10 mg/m^3 resulted in recovery of too few AMs for biochemical measurements; however after exposure to 1 mg/m^3, considerably fewer AMs were lost. These data indicated that in order to obtain sufficient numbers of AMs from exposed rats for nucleotide studies, the exposure concentrations must be less than 10 mg $CdSO_4$/m^3.

The EC values of AMs from rats exposed to 1 or 10 mg $CdSO_4$/m^3 was significantly decreased when measured immediately or 4 h after exposure. By 24 h after exposure, EC had returned to control levels. This return to control levels may have been due to an influx of uninjured AMs in response to lung injury, or to other steady state mechanisms operating intracellularly to maintain a high energy state in the AMs. It is not known whether this mechanism also exists *in vitro*.

Exposure to 10 mg/m^3 resulted in significant levels of cellular Cd that tended to decrease with time after exposure. The decrease in Cd levels in AMs from exposed rats with time after exposure could have been due to clearance of phagocytized Cd, or possibly to removal of Cd loosely bound to AMs by proteins released into the injured lung.

Exposure of rat and human AMs to $CdSO_4$ *in vitro* produced significant DNA damage, as measured by the alkaline elution technique. A comparison of human and rat AM cell sensitivity was not possible based upon this endpoint because only one experiment was performed with human AMs due to time constraints.

Additional information was obtained in these experiments by evaluating the lung lavage fluid for protein. Protein content of lung lavage fluid has been used in other studies as a short-term indicator of lung injury (Hu et al., 1982). A fourfold increase in lung lavage fluid protein was

detected immediately after exposure indicating acute damage to the lung. Lavage fluid protein increased by 13-fold to 685 µg/mL, typical of severe pulmonary edema. Cadmium toxicity to the lung was also indicated by the reduced recovery of lavage fluid with time after exposure. The decreased recovery of lavage fluid was probably due to the pulmonary edema and possibly constriction of the airways. Although the decreased recovery of lavage fluid may have contributed to the reduction in the numbers of AMs, the decrease in recovery of lavage fluid was much smaller (23%) than the decrease in AM numbers (92%).

In summary, significant progress was made in validating the proposed extrapolation model. The most significant finding was that *in vivo* respiratory tissue (NTE or AMs) contains less Cd following exposure than similar cells exposed *in vitro*. This finding made an *in vivo-in vitro* correlation of cell sensitivity very difficult since cellular doses were several orders of magnitude different. If *in vivo* and *in vitro* cellular doses could be made more comparable, a meaningful correlation 1 value could be obtained. Future work should begin by focusing on this problem. The determination of a preliminary value for correlation 2 is a noteworthy accomplishment, and experiments should be repeated to confirm this value. Future work should include logical extensions to these studies, such as determining cell sensitivity values for different species, tissues, and particulates. These correlation values could then be combined with tissue dosimetry data from ongoing EPA studies to determine relative exposure concentrations to achieve similar tissue responses. This extrapolation model should also allow accurate human risk assessments to be made from toxicology studies using cultured human cells. The studies reported herein have provided important groundwork toward this achievable goal.

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